IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: John L. Schenk, Allison C Lindsey

Application No.: 10/522,320

Filed: July 22, 2003

For: Sperm Cell Process System

Attorney Docket No.: XY-Optimum-USNP

Confirmation No.: 6962

Group Art Unit: 1657

Examiner: Gough, Tiffany Maureen

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE UNDER 37 C.F.R. § 1.10 FOR "EXPRESS MAIL"

Date of Deposit: December 13, 2010 Express Mail Label No.: EM527204329US

I hereby certify that this document along with Exhibits A-K are being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail post Office to Addressee" service under 37 C.F.R. § 1.10 and addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Lioudmila Townsend

DECLARATION UNDER 37 C.F.R. §1.131(a)

Dear Sir/Madam:

John L. Schenk and Allison C. Lindsey, the applicants in the above-identified patent application, declare as follows:

1. That sometime prior to March 22, 2002, John L. Schenk ("Schenk") and Allison C. Lindsey ("Lindsey") employees of XY, Inc. (now XY, LLC)("XY") the assignee of the above-identified patent application ("Assignee") conceived of the method of separating sperm cells which includes the steps of: obtaining semen from a species of mammal which contains a plurality

of sperm cells and incubating the semen prior to staining to maintain a temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase of between about 5°C and about 25°C, staining the sperm cells with a DNA binding stain for a period of time of about 25 minutes to about 60 minutes, thereafter determining a sex characteristic of a plurality of the sperm cells which allows separating the plurality of sperm cells based upon the sex characteristic into an X chromosome bearing population and a Y chromosome bearing population, and collecting at least one of the X chromosome bearing population and the Y chromosome bearing population (the "Invention") as described in the above-identified application (the "Application").

2. That sometime prior to March 22, 2002, Lindsey commenced reducing the Invention to practice by way of experimental trials to determine the effect of various factors during staining of sperm cells on sperm viability and resolution of sperm cells during separation into X chromosome bearing and Y chromosome bearing populations. Now produced and shown to me and marked Exhibit A is the document entitled "Prelim. Exp. 1--Seminal Plasma X Sperm Concentration During Staining" which evidences that Lindsey tested the effect of seminal plasma concentration and sperm cell concentration on sperm cells immediately after staining and after storage in a cold room for 18 hours (dates prior to March 22, 2002 redacted). Now produced and shown to me and marked Exhibit B are bar graphs which illustrate the results of Prelim. Exp. 1 (page 1, second column of graphs Seminal Plasma: Stallion A, Seminal Plasma: Stallion B, and Seminal Plasma: Stallion C) (dates prior to March 22, 2002 redacted).

3. That sometime prior to March 22, 2002, Lindsey continued to reduce the Invention to practice by way of further experimental trials to determine the effect of type of extender, seminal plasma concentration, and stain concentration on sperm viability and resolution of sperm cells during separation into X chromosome bearing and Y chromosome bearing populations. Now produced and shown to me and marked Exhibit C is the document entitled "Experiment 2: Extender, Seminal Plasma & Stain Concentration" which evidences the experimental design used to test the effect of extender type, seminal plasma concentration and stain concentration on sperm cells collected from a male mammal immediately after staining and after storage in a cold room for 18 hours (dates prior to March 22, 2002 redacted). Now produced and shown to me and marked Exhibit B are bar graphs which illustrate the results of Experiment 2 (entire document) (dates prior to March 22, 2002 redacted).

4. That sometime prior to March 22, 2002, Lindsey further continued to reduce the Invention to practice by way of experimental trials to determine the effect of staining pH, time, red food dye, and methods to stimulate motility on sperm viability and resolution of sperm cells during separation into X chromosome bearing and Y chromosome bearing populations. Now produced and shown to me and marked Exhibit D is the document entitled "Prelim. C Experiment 2" which evidences the experimental design used to test the effect of effect of staining pH, time, red food dye, and methods to stimulate motility on sperm cells collected from a male mammal immediately after staining and after storage in a cold room for 18 hours (dates prior to March 22, 2002 redacted). Now produced and shown to me and marked Exhibit E are bar graphs which illustrate the results of Prelim. C Experiment 2 (entire document) (dates prior to March 22, 2002 redacted).

5. That sometime prior to March 22, 2002, Lindsey further continued to reduce the Invention to practice by way of experimental trials to determine the effect of storage temperature on sperm cells prior to staining and separation into X chromosome bearing and Y chromosome bearing populations. Now produced and shown to me and marked Exhibit F is the document entitled "Proposed Protocol-Stallion Fertility Trial--A Comparison of the Effects of Shipping Temperature (5°C and 15°C) and Method of Insemination (Hysteroscopic or Rectally Guided) on the Fertility of Sex-Sorted Stallion Sperm" which evidences the experimental design used to test the effect of storage of sperm cells at either 5°C or 15°C prior to staining and sorting (dates prior to March 22, 2002 redacted).

6. That sometime prior to March 22, 2002, Lindsey summarized the results of several in vivo studies to determine the effects of type of media, shipping temperature, sperm concentration during staining, stain concentration during staining, pH during staining, osmolality during staining, length of staining, and sperm assay methods. Now produced and shown to me and marked Exhibit G is the document entitled "Summary of Equine Trials" which evidences the experimental design and results of several experiments including Experiment 2c which evidences that sperm samples stained for less than 60 minutes contained fewer percent dead and exhibited better resolution than those stained for 60 minutes." and Experiment 2d which evidences that the

temperature at which sperm cells stored has an effect the post storage and post stain motility and percent dead of sperm cells (dates prior to March 22, 2002 redacted).

7. That sometime prior to March 22, 2002, the results of the experiments described in Paragraphs 2-6 of this Declaration were reduced to articles and submitted to Equine Veterinary Journal for publication. Now produced and shown to me and marked Exhibit H is a document entitled "Facsimile Transmittal" which evidences that Lindsey submitted corrected proofs of the articles entitled "Hysteroscopic Inseminaton of Low Numbers of Flow Sorted Fresh and Frozen/Thawed Stallion Spermatazoa" and "Hysteroscopic Insemination of Mares With Low Numbers of Nonsorted or Flow Sorted Spermatazoa" each cited in the Office Action relating to the Application mailed June 23, 2010 on page 4. Now produced and shown to me and marked Exhibit I is the proof of the journal article entitled "Hysteroscopic Inseminaton of Low Numbers of Flow Sorted Fresh and Frozen/Thawed Stallion Spermatazoa" corrected by Lindsey. Now produced and shown to me and marked Exhibit J is the proof of the journal article entitled "Hysteroscopic Insemination of Mares With Low Numbers of Nonsorted or Flow Sorted Spermatazoa" corrected by Lindsey (dates prior to March 22, 2002 redacted).

8. That during the period between submission of the articles to Equine Veterinary Journal and July 22, 2002, Lindsey provided information to attorney Craig Miles ("Miles") then employed by Santangelo Law Offices ("SLO") for the preparation of a United States Provisional Patent Application.

9. That Miles prepared and filed the United States Provisional Patent Application with the USPTO which was assigned Serial No. 60/400,486 and a filing date of July 22, 2002 (the '486 Application". Now produced and shown to me and marked Exhibit K is a copy of the '486 Application as filed July 22, 2001.

10. That each of Lindsey and Schenk believe that this Declaration along with the cited Exhibits provide evidence which is of a character and weight, that establishes a reduction to practice of the Invention prior to the effective date of the Allison references (Equine Vet. Journal, March 2002, p. 128-132 and Equine Vet. Journal, March 2002, p. 121-127) cited by the United States Patent and Trademark Office in the Office Action mailed June 23, 2010, or

conception of the Invention prior to the effective date of the Allison references coupled with due diligence from prior to said effective date to a subsequent reduction to practice or to the filing of the '486 Application.

11. That all the acts of conception and reduction to practice occurred after December 8, 1993 in the United States, a NAFTA country, or a WTO member country.

12. The Declarants further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment or both, under Section 1001 of Title 18 of the United States Code and any such willful false statement may jeopardize the validity of this application or any patent resulting therefrom.

Dated this 3 day of December, 2010

Allison C. Lindsey/Inventor

John L. Schenk/Inventor

Assignee, XY, LLC

By: _____
Thomas Gilligan, General Manager

conception of the Invention prior to the effective date of the Allison references coupled with due diligence from prior to said effective date to a subsequent reduction to practice or to the filing of the '486 Application.

11. That all the acts of conception and reduction to practice occurred after December 8, 1993 in the United States, a NAFTA country, or a WTO member country.

12. The Declarants further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment or both, under Section 1001 of Title 18 of the United States Code and any such willful false statement may jeopardize the validity of this application or any patent resulting therefrom.

Dated this 6th day of December, 2010

Allison C. Lindsey/Inventor

John L. Schenk/Inventor

Assignee, XY, LLC

By: _____
Thomas Gilligan, General Manager

Staining Optimization Trial
Prelim. Exp. 1

Seminal plasma x sperm concentration during staining

Objectives:

1. To determine if additional seminal plasma during staining has an effect on viability or resolution enough to warrant inclusion in a more in-depth, closely controlled trial.

2. To determine if sperm concentration during staining has an effect on viability or resolution enough to warrant inclusion in a more in-depth, closely controlled trial.

Scope:

A. 3 stallions
   a. Dunn Cutter Dandy Boy
   b. Sylekt
   c. Scotti
B. 2 seminal plasma concentrations during staining
   a. No additional seminal plasma
   b. 10% additional seminal plasma during staining
C. 3 sperm concentrations
   a. 50×10^6 sperm/mL
   b. 150×10^6 sperm/mL
   c. 450×10^6 sperm/mL

Design:

[redacted]

1:00pm
- A. Collect stallions
- B. Evaluate sperm for volume, concentration, and motility
- C. Remove 3ml from each raw ejaculate for seminal plasma preparation.
  - a. Aliquot the semen into equal volumes of 1.5 ml in 2 ml Eppendorf tubes
  - b. Centrifuge the raw semen for 20 min. using the Beckman Microfuge E centrifuge
  - c. Carefully pour the supernatant from each tube and pool volumes
  - d. Filter the seminal plasma into 2-2ml Eppendorf tubes using a .22 micron syringe filter
  - e. Required seminal plasma volume for each stallion/day is 300µl
  - f. Store 1 tube of seminal plasma from each stallion in cold room for 18h evaluation
- D. Extend the remainder of ejaculates to 25×10^6 sperm/mL in KMT

EXHIBIT A

a. Package 2 bags/stallion for evaluation at 18h
b. 40 ml in whirlpak, room temp

1:45pm E. Centrifuge remaining ejaculate at 600g, 10 min.
a. 40 ml in each 50 ml conical tubes
b. No less than 3 tubes per stallion

F. Aspirate supernatant to ~1.5 ml per tube

G. Gently resuspend pellet with transfer pipettes (pellets are very soft)

H. Pool all pellets from similar stallions and mix thoroughly

I. Count hemacytometers for each stallion (3 stallions)
a. 230µl kill solution: 10µl sperm
b. 4 chambers/stallion

3:00pm J. Prepare staining tubes for each stallion as follows:
a. 50×10^6 sperm/mL, No additional seminal plasma
b. 150×10^6 sperm/mL, No additional seminal plasma
c. 450×10^6 sperm/mL, No additional seminal plasma
d. 50×10^6 sperm/mL, 10% additional seminal plasma
e. 150×10^6 sperm/mL, 10% additional seminal plasma
f. 450×10^6 sperm/mL, 10% additional seminal plasma
1. For 10% seminal plasma treatments, replace 100µl of required extender with filtered seminal plasma

K. Incubate all samples for 1h in water bath

4:00pm L. Add 1 ml extender with food dye (warmed) to all stained samples

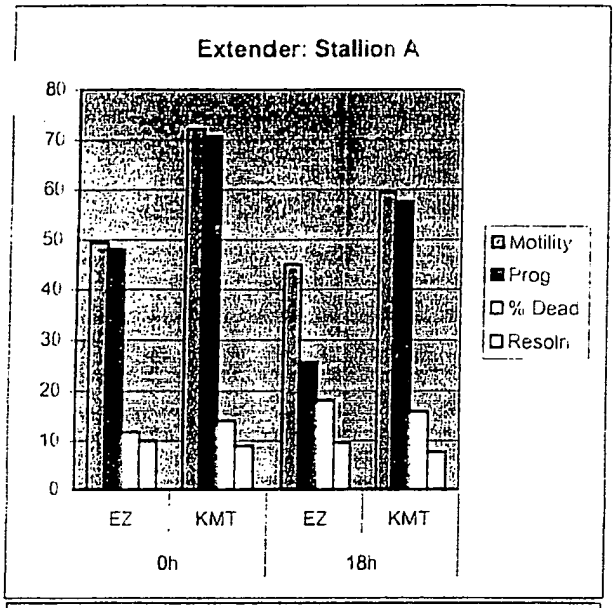
M. Filter samples

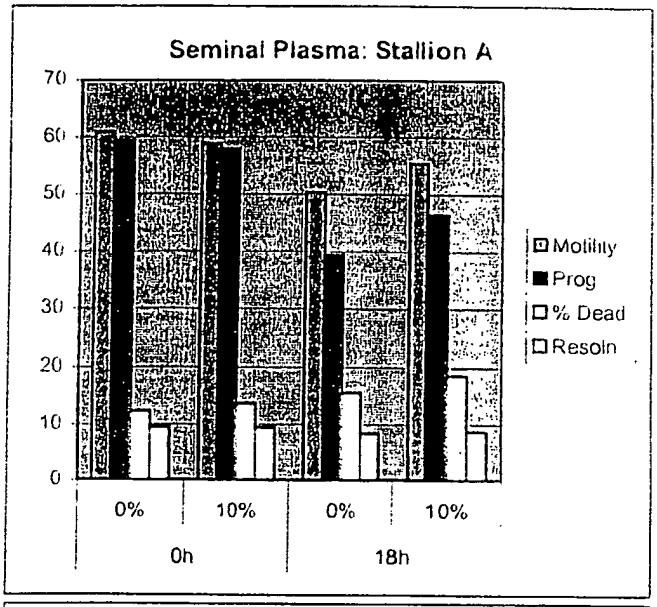
N. Evaluate sperm samples for motility, live/dead, and resolution
a. Dilute samples in EZ Mixin for motility evaluation
i. 70µl EZ Mixin: 10µl sperm
b. Run samples on flow for live/dead analysis and resolution score
i. Subjectively analyze the resolution of each sample – minor tuning may be required.
ii. Score the resolution on a scale of 1-10.
1. 1 = Best
2. 5 = Flat
3. 10 = Poor

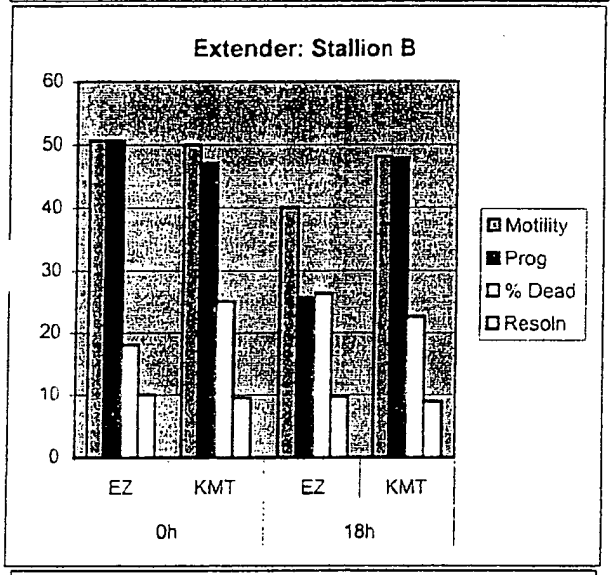
[redacted]

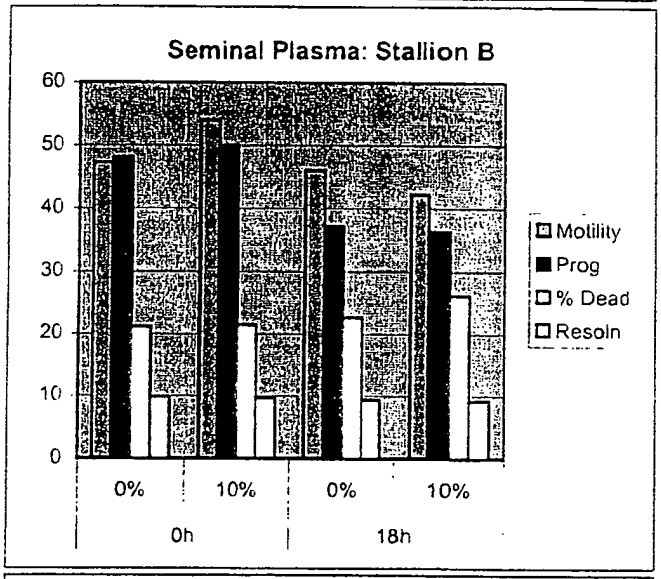
8:00am Repeat steps E thru N using the stored sperm samples
Samples should be ready to run at 10:15. If this presents a sorter conflict, the samples could be processed at 1:00 Friday, to run at 4:00. It should be realized, however, that 24h storage could greatly affect viability and motility when compared to 18h storage.

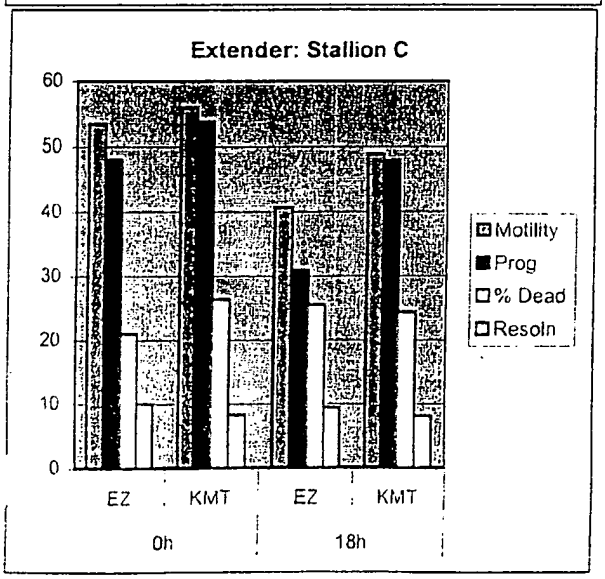
10%

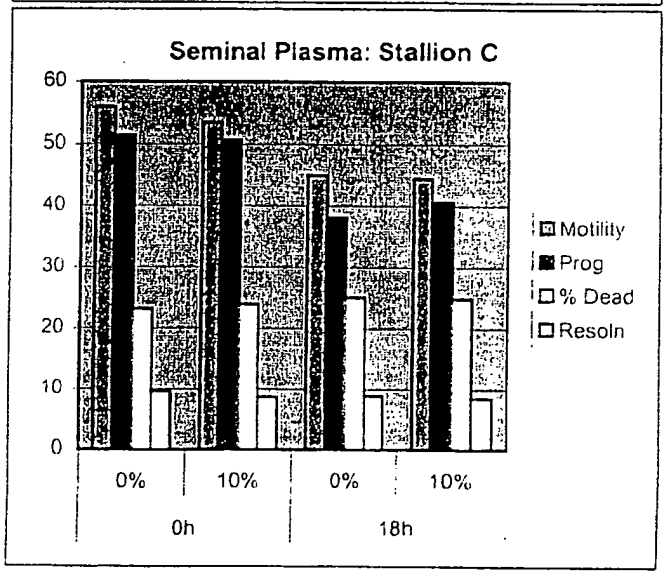






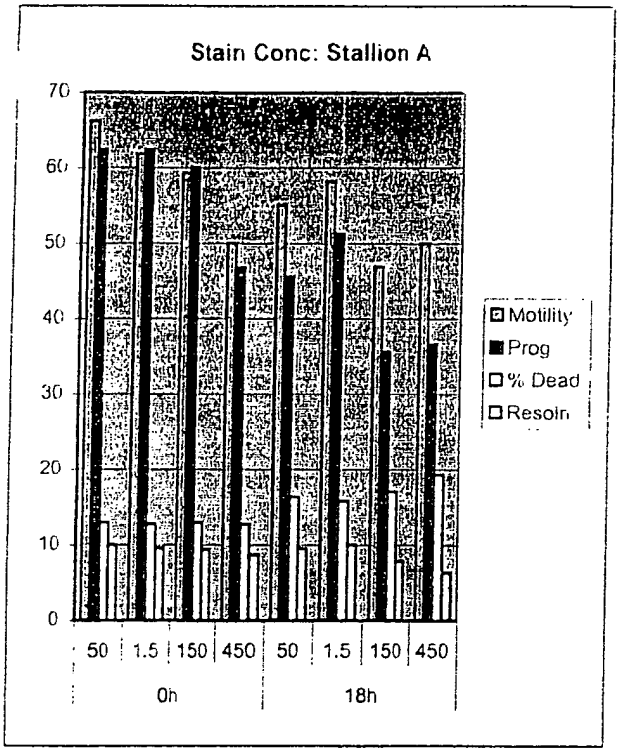


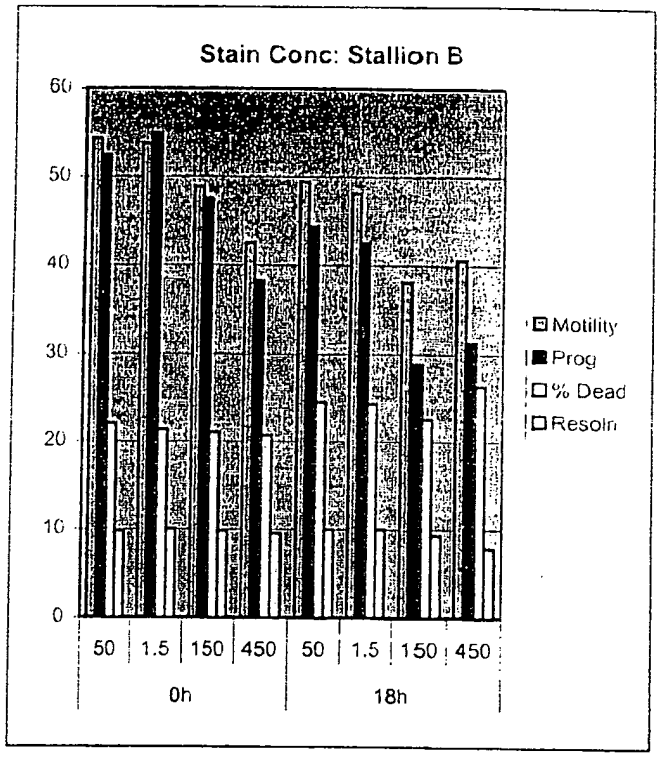


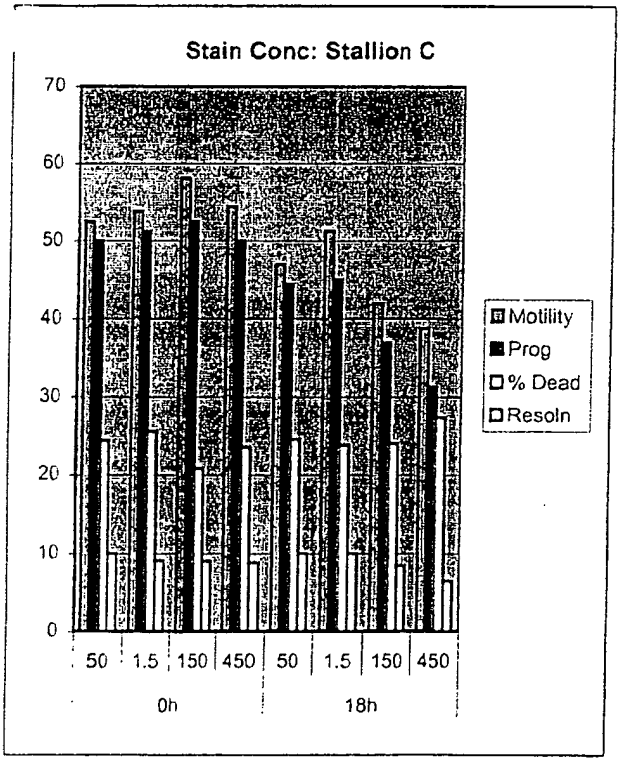


TKS [redacted]

EXHIBIT B


Stain Conc: Stallion A
70
60
50
40
30
20
10
0
50 1.5 150 450 50 1.5 150 450
0h
18h
Motility
Prog
% Dead
Resoln


Stain Conc: Stallion B
60
50
40
30
20
10
0
50 1.5 150 450 50 1.5 150 450
0h
18h
Motility
Prog
% Dead
Resoln


Stain Conc: Stallion C
70
60
50
40
30
20
10
0
50 1.5 150 450 50 1.5 150 450
0h
18h
Motility
Prog
% Dead
Resoln

Experiment 2: Extender, seminal plasma & stain concentrations

Stallion A=Sylekt, B=Scottie, C=Dum

Tube #	Semen	Stallion	Code #	Extender	Seminal	Stain	Total Motility		Progressive Motility		% Dead	Resolution
					Plasma	Conc	Obser 1	Obser 2	Obser 1	Obser 2		
1	Fresh	A	1	EZ	0	50	60	65	60	60	13	10
2	Fresh	A	2	EZ	0	1.5	50	35	50	50	11	10
3	Fresh	A	3	EZ	0	150	55	60	55	55	10	10
4	Fresh	A	4	EZ	0	450	35	35	35	35	11	10
5	Fresh	A	5	EZ	10	50	45	65	45	45	12	10
6	Fresh	A	6	EZ	10	1.5	60	60	60	60	12	10
7	Fresh	A	7	EZ	10	150	40	30	40	40	12	10
8	Fresh	A	8	EZ	10	450	40	55	40	40	13	10
9	Fresh	A	9	KMT	0	50	70	80	70	70	12	10
10	Fresh	A	10	KMT	0	1.5	70	80	70	70	12	10
11	Fresh	A	11	KMT	0	150	70	75	70	70	15	10
12	Fresh	A	12	KMT	0	450	65	70	65	65	14	6
13	Fresh	A	13	KMT	10	50	75	70	75	75	15	10
14	Fresh	A	14	KMT	10	1.5	70	70	70	70	16	8.5
15	Fresh	A	15	KMT	10	150	75	70	75	75	15	7.5
17	Fresh	B	1	EZ	0	50	50	50	50	50	17	10
18	Fresh	B	2	EZ	0	1.5	55	30	55	55	18	10
19	Fresh	B	3	EZ	0	150	60	35	55	55	15	10
20	Fresh	B	4	EZ	0	450	35	55	35	35	17	10
21	Fresh	B	5	EZ	10	50	60	60	60	60	24	10
22	Fresh	B	6	EZ	10	1.5	65	65	65	65	20	10
23	Fresh	B	7	EZ	10	150	55	60	45	45	15	10
24	Fresh	B	8	EZ	10	450	40	35	40	40	18	10

EXHIBIT C

25	Fresh	B	9	KMT	0	50	55	60	55	55	23	10
26	Fresh	B	10	KMT	0	1.5	55	50	55	55	22	10
27	Fresh	B	11	KMT	0	150	40	35	40	40	30	10
28	Fresh	B	12	KMT	0	450	40	50	40	40	27	8.5
29	Fresh	B	13	KMT	10	50	45	55	45	45	24	9
30	Fresh	B	14	KMT	10	1.5	45	65	45	45	25	10
31	Fresh	B	15	KMT	10	150	50	55	50	50	24	9
32	Fresh	B	16	KMT	10	450	50	55	50	50	24	9
33	Fresh	C	1	EZ	0	50	40	45	40	40	23	10
34	Fresh	C	2	EZ	0	1.5	55	60	55	55	23	10
35	Fresh	C	3	EZ	0	150	50	65	40	40	21	10
36	Fresh	C	4	EZ	0	450	50	60	50	50	19	10
37	Fresh	C	5	EZ	10	50	50	60	50	50	21	10
38	Fresh	C	6	EZ	10	1.5	55	50	55	55	20	10
39	Fresh	C	7	EZ	10	150	50	60	50	50	21	10
40	Fresh	C	8	EZ	10	450	45	60	45	45	20	10
41	Fresh	C	9	KMT	0	50	55	60	55	55	29	10
42	Fresh	C	10	KMT	0	1.5	50	60	50	50	29	10
43	Fresh	C	11	KMT	0	150	65	65	65	65	14	10
44	Fresh	C	12	KMT	0	450	55	60	55	55	27	7
45	Fresh	C	13	KMT	10	50	55	55	55	55	Plug 2x	
46	Fresh	C	14	KMT	10	1.5	45	55	45	45	30	6.5
47	Fresh	C	15	KMT	10	150	55	55	55	55	27	6
48	Fresh	C	16	KMT	10	450	50	55	50	50	28	8

49	18h	A	1	EZ	0	50	50	20	30	5	15	10
50	18h	A	2	EZ	0	1.5	55	30	30	15	14	10
51	18h	A	3	EZ	0	150	40	10	25	0	13	10
52	18h	A	4	EZ	0	450	40	60	25	10	23	8
53	18h	A	5	EZ	10	50	55	55	55	25	20	10
54	18h	A	6	EZ	10	1.5	55	65	55	50	19	10
55	18h	A	7	EZ	10	150	40	55	25	10	23	9
[redacted]												
57	18h	A	9	KMT	0	50	70	75	70	75	13	9
58	18h	A	10	KMT	0	1.5	70	65	70	65	13	10
59	18h	A	11	KMT	0	150	60	60	60	60	15	5
60	18h	A	12	KMT	0	450	50	50	50	40	18	5
61	18h	A	13	KMT	10	50	60	55	60	45	17	9
62	18h	A	14	KMT	10	1.5	60	65	60	65	17	10
63	18h	A	15	KMT	10	150	55	55	50	55	17	7.5
64	18h	A	16	KMT	10	450	50	50	45	50	17	6
65	18h	B	1	EZ	0	50	40	50	30	20	28	10
66	18h	B	2	EZ	0	1.5	40	45	25	15	23	10
67	18h	B	3	EZ	0	150	30	25	20	5	22	10
68	18h	B	4	EZ	0	450	30	60	15	50	24	9
69	18h	B	5	EZ	10	50	40	55	40	55	26	10
70	18h	B	6	EZ	10	1.5	40	40	40	40	31	10
71	18h	B	7	EZ	10	150	30	45	10	20	25	10
72	18h	B	8	EZ	10	450	25	45	10	15	31	8.5
73	18h	B	9	KMT	0	50	50	60	50	60	20	10
74	18h	B	10	KMT	0	1.5	50	65	50	65	20	10
75	18h	B	11	KMT	0	150	40	60	40	60	20	9
76	18h	B	12	KMT	0	450	40	50	40	50	24	7

77	18h	B	13	KMT	10	50	50	50	50	50	24	10
78	18h	B	14	KMT	10	1.5	45	60	45	60	23	10
79	18h	B	15	KMT	10	150	40	35	40	35	23	8
80	18h	B	16	KMT	10	450	35	40	30	40	26	7
81	18h	C	1	EZ	0	50	35	45	30	30	26	10
82	18h	C	2	EZ	0	1.5	40	50	20	20	25	10
83	18h	C	3	EZ	0	150	35	60	30	40	24	10
84	18h	C	4	EZ	0	450	40	50	30	50	26	8
85	18h	C	5	EZ	10	50	40	45	40	45	26	10
86	18h	C	6	EZ	10	1.5	45	55	45	55	24	10
87	18h	C	7	EZ	10	150	30	15	25	5	25	10
88	18h	C	8	EZ	10	450	35	30	20	10	28	7
89	18h	C	9	KMT	0	50	45	50	45	50	24	10
90	18h	C	10	KMT	0	1.5	50	55	50	55	24	10
91	18h	C	11	KMT	0	150	45	45	45	45	23	7.5
92	18h	C	12	KMT	0	450	35	40	30	40	28	6
93	18h	C	13	KMT	10	50	50	65	50	65	22	10
94	18h	C	14	KMT	10	1.5	55	60	55	60	22	10
95	18h	C	15	KMT	10	150	45	60	45	60	24	6
96	18h	C	16	KMT	10	450	40	40	35	35	27	5

[redacted]

Prelim. C, Experiment #2

Staining pH, time, red food dye, and methods to stimulate motility

Objectives:

1. To determine if there is a trend for sperm stained in a more basic pH for 30 or 60 minutes to resolve more completely into X and Y populations.

2. To determine how much red food dye is necessary in the stained samples to drop the dead population to a desired location on the flow histogram.

3. To determine the most effective method to stimulate the motility of stored, stained stallion sperm.

Scope:

A. 3 stallions
   a. Rowdy
   b. Sylekt
   c. Gunsmoke

B. 2 pH levels during staining
   a. 7.1
   b. 7.9

C. 4 levels of red food dye (added 1:1 with stained sample)
   a. 2.5µl/mL 5%– final conc. 1.25µl/mL 5%
   b. 2.0µl/mL 5%– final conc. 1.00µl/mL 5%
   c. 1.5µl/mL 5%– final conc. 0.75µl/mL 5%
   d. 3µl/mL 2% - final conc. 1.5µl/mL 2%

D. 2 chemicals to stimulate motility
   a. Caffeine (begin at 2mM, 5 min.)
   b. NaPyruvate (begin at 2.5mM, 5 min.)

E. 2 staining times at both pH
   a. 30 min
   b. 60 min

F. 3 responses
   a. Resolution (B only)
   b. Motility (B and D only)
   c. Percent Dead (B and C only)

Design:

1:00pm
   A. Collect stallions
   B. Evaluate sperm for volume, concentration, and motility
   C. Extend the remainder of ejaculates to 25 x 10^6 sperm/ml in KMT
      a. We need 120 ml of extended semen per stallion
      b. 40 ml in each whirlpak bag

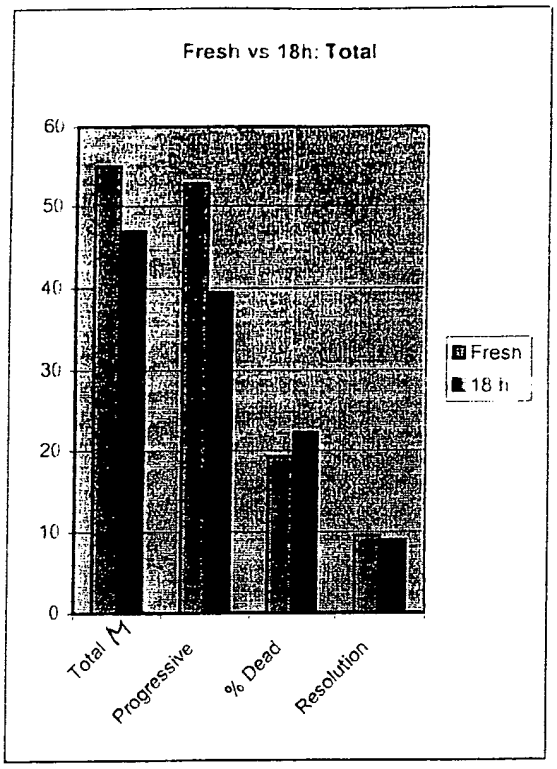
[redacted]

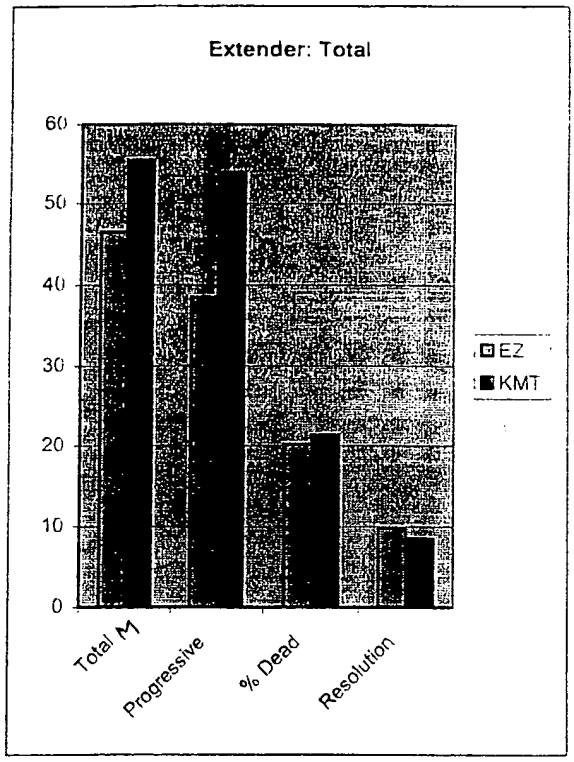
ACL

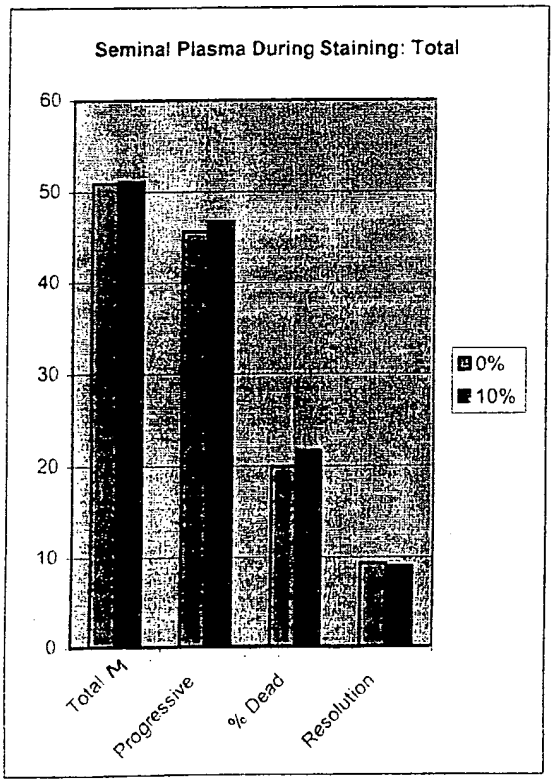
EXHIBIT D

[redacted]

	D.	Store sperm for ~18h at room temp.
8:00am	E.	Unpackage samples and centrifuge 600g, 10 min.
	F.	Aspirate supernatant to ~1.5 ml per tube
	G.	Gently resuspend pellet with transfer pipettes (pellets are very soft)
	H.	Pool all pellets from similar stallions and mix thoroughly
8:30am	I.	Count hemacytometers for each stallion (3 samples)
		a. 2390 µl kill solution: 10µl sperm
		b. 4 chambers/sample
9:30am	J.	Extend all samples to 400 x 10^6 sperm/ml with KMT
	K.	Prepare staining tubes for each stallion as follows:
		a. All tubes: 200 x 10^6 sperm/mL, 1ml volume, 12.4 µl HO
		b. Prepare 4 tubes of each stallion at pH 7.1
		c. Prepare 4 tubes of each stallion at pH 7.9
10:30am	L.	Incubate all samples for 1h in water bath at 34°C
11:30am	M.	Add 1 ml KMT with food dye (warmed) to all stained samples
		a. One tube of each treatment/stallion should be extended with the following:
		i. 1ml KMT with 2.5µl/ml 5% red food dye
		ii. 1ml KMT with 2.0µl/ml 5% red food dye
		iii. 1ml KMT with 1.5µl/ml 5% red food dye
		iv. 1ml KMT with 3µl/ml 2% red food dye
		b. Extender should be place in water bath ~45 minutes after the beginning of staining
	N.	Filter samples
	O.	Evaluate sperm samples for motility, live/dead, and resolution (12 samples)
		a. Dilute samples in KMT for motility evaluation
		i. 140µl KMT: 20µl sperm, 5 min.
		ii. 140µl KMT with 2mM Caffeine: 20µl sperm, 5 min.
		iii. 140µl KMT with 2.5mM NaPyruvate: 20µl sperm, 5 min.
		b. Run samples on flow for live/dead analysis and resolution score
		i. Subjectively analyze the resolution of each sample – minor tuning may be required.
		iv. Score the resolution on a scale of 1-10.
		1. 1 = Best
		2. 5 = Flat
		3. 10 = Poor


Fresh vs 18h: Total
60
50
40
30
20
10
0
Total M
Progressive
% Dead
Resolution
Fresh
18 h


Extender: Total
60
50
40
30
20
10
0
Total M
Progressive
% Dead
Resolution
EZ
KMT


Seminal Plasma During Staining: Total
60
50
40
30
20
10
0
Total M
Progressive
% Dead
Resolution
0%
10%

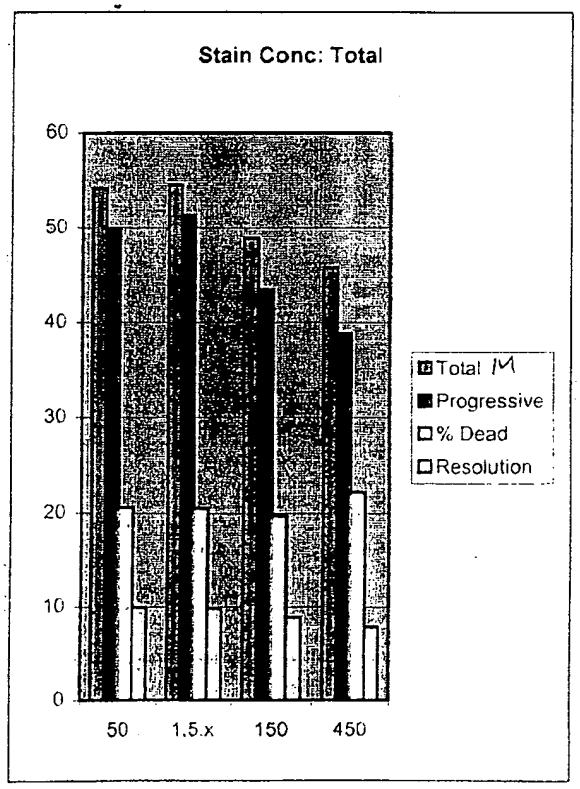
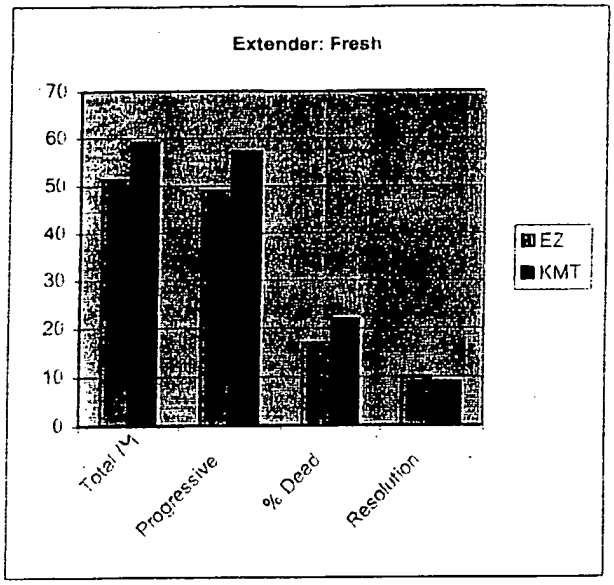

Stain Conc: Total
60
50
40
30
20
10
0
50
1.5.x
150
450
Total M
Progressive
% Dead
Resolution

EXHIBIT E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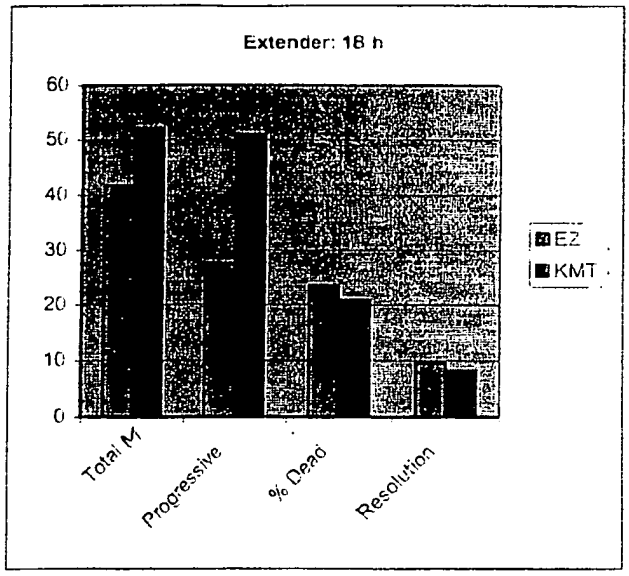
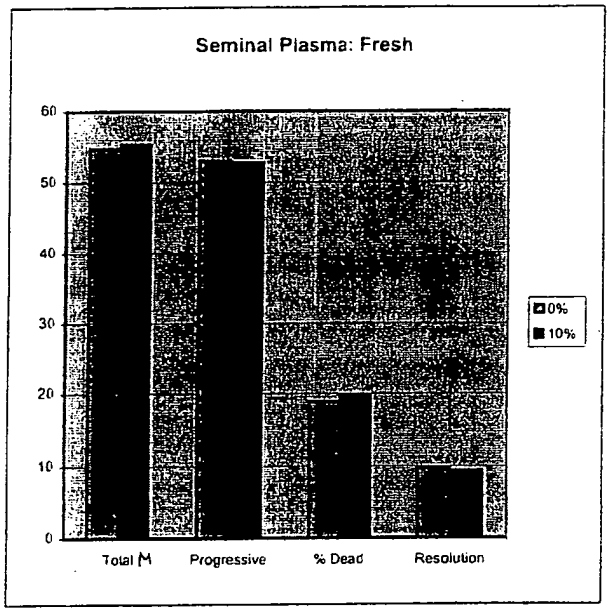
60
50
40
30
20
10
0
EZ
KMT
Total M
Progressive
% Dead
Resolution


Extender: 18 h
60
50
40
30
20
10
0
EZ
KMT
Total M
Progressive
% Dead
Resolution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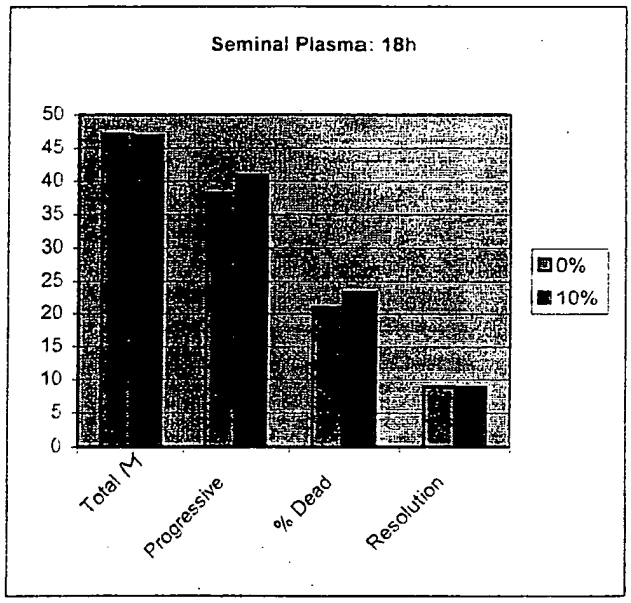
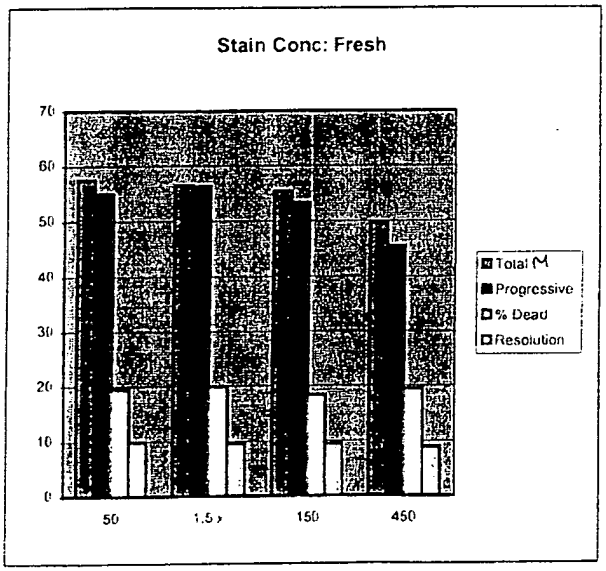
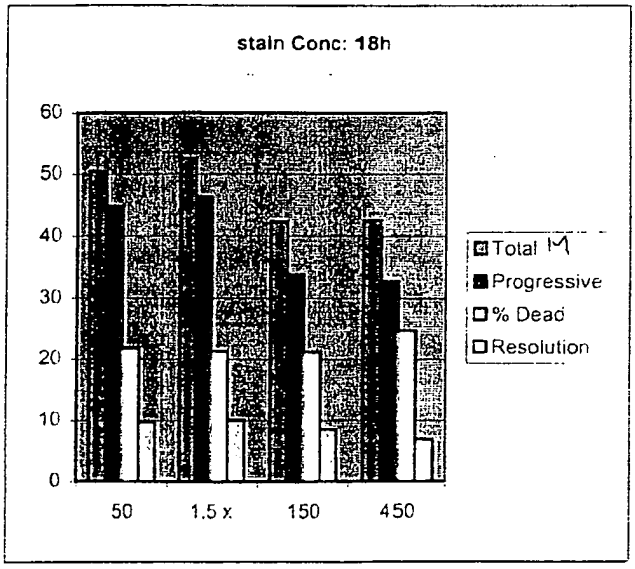
60
50
40
30
20
10
0
0%
10%
Total M
Progressive
% Dead
Resolution


Seminal Plasma: 18h
50
45
40
35
30
25
20
15
10
5
0
0%
10%
Total M
Progressive
% Dead
Resolution


Stain Conc: Fresh
70
60
50
40
30
20
10
0
Total M
Progressive
% Dead
Resolution
50
1.5 x
150
450


stain Conc: 18h
60
50
40
30
20
10
0
Total M
Progressive
% Dead
Resolution
50
1.5 x
150
450

Proposed Protocol – Stallion Fertility Trial [redacted]

A comparison of the effects of shipping temperature (5°C and 15°C) and method of insemination (hysteroscopic or rectally guided) on the fertility of sex-sorted stallion sperm.

Goals:

1. To compare the fertility of sorted stallion sperm when processed immediately after shipment at either 5°C or 15°C.
2. To compare pregnancy rates obtained by inseminating low numbers of sperm (20 x 10^6 total sorted sperm) using either a video-endoscopic method or rectally guided method for deep uterine insemination.

Background:

1. Based on in vitro work completed Spring 2001, it has been found that stallion sperm maintain similar post-sort motility following 18h shipment at both 15°C and 5°C. We would like to determine if the fertility of sperm shipped at these two temperatures is comparable.
2. In a trial conducted at Texas A&M using 5 x 10^6 24h cooled sperm from a single stallion, no difference in pregnancy rates was found when comparing video-endoscopic insemination and rectally-guided insemination. We would like to determine if these results are repeatable when sex-sorted sperm are utilized.

Animal specifics:

1. Mares – ~30 mares are available. I suggest attempting no less than 60 cycles on this experiment, with no less than 20 inseminations per treatment group. All mares will be synchronized for insemination in each of three 6-day periods.
2. Stallions – 2 stallions should be used. An attempt will be made to balance each treatment group with regard to stallion and sex ratio. Rowdy and Durn Cutter Dandy Boy will be used for this trial.

Proposed treatment groups:

1. Sperm sorted following 18h storage at 5°C, 20 × 10^6 sperm, hysteroscopic insemination, 30h post-hCG.
2. Sperm sorted following 18h storage at 5°C, 20 × 10^6 sperm, rectally-guided insemination 30h post-hCG.
3. Sperm sorted following 18h storage at 15°C, 20 × 10^6 sperm, hysteroscopic insemination 30h post-hCG.

5°C Hyst
~~5 R~~
15°C Hyst
15°C Rect

EXHIBIT F

Detailed Protocol

Scope:

A. 2 stallions
B. No less than 60 mare cycles
C. 2 shipping temperatures
   a. 5°C
   b. 15°C
D. 2 insemination methods
   a. Videoendoscopic insemination
   b. Rectally-guided insemination
E. 4 responses
   a. Pregnancy rate
   b. Motility
      1. Post-sort
   c. Stimulated Motility – 2mM Caffeine
      1. Post-sort
   d. Percent Dead

I. STALLION COLLECTION AND EJACULATE ASSESSMENT

1. Collect ejaculates from 1 or 2 stallions/day
2. Evaluate semen for volume, concentration, and motility
   A. Record ejaculate volume to the nearest mL.
   B. Pour off a 1 mL working sample to be used for evaluation
   C. Visually estimate the percentage of progressively motile sperm.
      a. Extend a portion of raw semen 20:1 with EZ Mixin
         i. Add 0.25 mL raw sement to 4.75 mL EZ Mixin CST extender
         ii. Gently invert tube several times to mix
      b. Examine under microscope to determine total and progressive motility
      c. Estimate each motility in increments of 5 percentage units
   D. Determine sperm concentration via Densimeter
      a. Zero the densimeter
         i. Add 3.42 mL formalin-saline to cuvette to zero machine.
      b. Dilute semen in formalin-saline 20:1 (formalin-saline:semen).
         i. Add 180 µl raw semen to cuvette containing formalin- saline
      c. Place diluted sample in densimeter and record concentration.

II. SHIPPED SAMPLE PREPARATION

1. Extend the remainder of ejaculate in CatKMT (24 U/ml BL catalase) to 25 x 10^6 sperm/ml
   A. Semen should be extended in a prewarmed flask

B. (Volume of ejaculate x raw concentration) = final extended volume
25 x 10^6 sperm/ml

C. Add items to a warm flask in the order listed below:
   a. Buffer
   b. Semen

2. Use parafilm to seal the flask
3. Gently invert flask 4 times (180° rotation) for sample mixing
4. Aliquot extended semen into labeled whirlpak bags in a volume of 40 ml/bag
5. Bags should be labeled with the following information:
   A. Stallion identification
   B. Storage temperature (5 or 15)
      a. Storage temperature will depend on # of inseminations and treatment groups each day
   C. Date
   D. Time of packaging
6. Place samples for 5° storage in an equitainer with 2 coolant cans
7. Place samples for 15° storage in a water bath placed in a cold room
8. Store samples for **18h**
   a. Keep samples protected from light
   b. Avoid any fluctuation in temperature for either treatment

III. UNPACKAGING AND RECONCENTRATION OF SAMPLES

1. Remove all samples from controlled temperatures
2. Place samples in a water bath at ambient temperature for 30 minutes
3. Allow 15 min for equilibration once samples have reached room temperature
4. Unpackage samples and transfer to 50-mL weighed tubes
   A. Aliquot sperm for post-shipping motility evaluation
      a. Using a transfer pipette, remove leftover semen from the bottom of whirlpak bags.
      b. Pool semen from similar treatments in a 5-ml tube and place in warm block.
5. Place 50-mL tubes in centrifuge, 600g, 10 min
6. Evaluate post-shipping motility
   A. Motility should be evaluated and recorded ~5 min after placement in warm block
7. Remove samples from centrifuge and aspirate samples to ~1.5ml
   A. Take care not to disrupt the pellet as tubes are removed from the centrifuge
8. Gently resuspend each pellet with transfer pipettes (pellets are very soft)
9. Pool pellets from similar treatments
10. Prepare hemacytometers for each shipping treatment (2)
    A. 2390 µl kill solution: 10µl sperm
    B. Count 4 chambers/sample
11. Extend sperm samples to 400 x 10^6 sperm/mL

IV. PREPARATION OF STAINING SAMPLES

1. Prepare staining samples as follows:
2. 2 ml staining samples
3. 100 x 10^6 sperm/mL
4. 3 levels of stain per shipping treatment
   - A. 8.68 µl Hoechst 33342
   - B. 10.54 µl Hoechst 33342
   - C. 12.44 µl Hoechst 33342
     - a. Hoechst 33342 is light sensitive and can be activated by fluorescence lighting. Therefore the stock solution and sample preparation should be protected from fluorescence lighting by enclosing container in aluminum foil or storing in an amber bottle.
     - b. Latex gloves should be worn when handling Hoechst 33342
     - c. Dry outside of pipette tip after aspiration by wiping with a Kimwipe.
     - d. Rinse pipette tip by aspirating and expelling Hoechst into buffer.
5. Staining buffer (KMT) and Hoechst should be combined and mixed vigorously prior to the addition of sperm
6. Sperm should be added immediately prior to placement in water bath
7. Cap sample tubes with polyethylene caps.
8. Gently invert tubes 4 times (180° rotation) for sample mixing.
9. Place samples in circulating water bath at 34° C
10. Place KMT with food dye in water bath
11. Remove samples from water bath
12. Add KMT with food dye (warmed) to all stained samples
    - A. Add 0.66 ml pink KMT to all samples
    - B. Concentration for flow evaluation should be 75 x 10^6 sperm/mL for all samples.
    - C. Final red dye concentration in all samples should be 0.75µl/ml 5% red food dye
    - D. Extender should be warmed to 34° C prior to addition to samples
13. Filter samples using yellow partec filters
14. Evaluate motility of stained samples and record
15. Incubate samples at 20-22 °C until use
16. Fresh samples should be prepared every hour using the level of stain that produces the best resolution

V. SORT TUBE PREPARATION

1. Determine 50-ml Falcon tube empty mass by weighing the tube and cap.
   - A. **Permanently record the weight on the 50-ml Falcon tube and on the cap.**
2. Deposit 4 ml of CatKMT (144 U/ml BL catalase), warmed to 20 – 22 °C to each of two 50 ml Falcon tubes
   - A. Label each 50-ml Falcon tube with the following information:
     - a. **Stallion identification**
     - b. **X- or Y-sort**
     - c. **An ascending numerical number**

3. Warm CatKMT catch buffer only as needed. This will aid in minimizing microbial growth.

VI. SORTING PROCEDURE:

1. Set sorting gates for 90% purity.
2. Sort both X and Y-bearing sperm.
3. Sort into 50-ml Falcon tubes containing 4-ml CatKMT extender.
4. Sheath fluid will be Z fluid (7.2 pH, 315 mOsm)
5. Record MoFlo parameters:
   A. Laser output (mW), amps
   B. Sort mode
   C. Drop drive frequency
   D. Drop drive amplitude
   E. Charge
   F. Plate deflection
   G. Total population coefficient of variation (CV)
   H. X and Y population coefficient of variation (cvs)
   I. Presence or absence of split between X and Y populations
   J. Percentage dead sperm
   K. Sort rate
   L. Abort rate
   M. Total number sorted
   N. Any sort problems
6. **Gently swirl Falcon tube every 500,000 sperm to mix sperm/catch fluid solution.**
   A. Mixing every 30 minutes is also acceptable.
7. Sorted sperm concentration ~800,000/ml.
   A. Sort into the 50-ml Falcon tube for a maximum of 2 h or a maximum of 30-ml of total volume.
   B. Volume will depend on number of inseminations, time, and treatment needs
   C. 20×10^6 sorted sperm needed per insemination
8. Combine tubes of corresponding sex from all machines.
9. Remove 1 mL from each pooled sample (X-bearing and Y-bearing).
10. Perform resort analysis on X's and Y's.
11. Discard sorted sperm is purity is less than 85%.

VII. CENTRIFUGATION:

1. Program the Eppendorf centrifuge, Model #5810 R.
   A. Turn the centrifuge ON and close the lid of the centrifuge.
   B. Program the centrifuge as follows:
      a. Temperature set at 22°C.
      b. g-force set at 850 x g.
      c. The g-force will appear as ***850** on the centrifuge display.
      d. Time set at 20 minutes.

2. Place the 50 ml Falcon tubes containing the sorted semen into a room temperature (22°C) centrifuge.
3. Make sure the sample tubes are balanced prior to centrifugation.
4. Centrifuge the sorted samples contained in the 50-ml Falcon tubes for 20 minutes at 850 x g.
   A. If volume in tubes is ≥ 30 mL, spin for 23 min.
   B. If volume in tubes is < 30 mL, spin for 20 min.
   C. Avoid spinning volumes less than 20 mL.

VIII. SAMPLE ASPIRATION:

1. Carefully remove only one 50-ml Falcon tube at a time from the centrifuge for aspiration
2. Aspirate the supernatant leaving a 100µl sperm pellet.
3. Cap each 50 ml centrifuge tube.
4. Be sure to aspirate all tubes before proceeding to next step.

IX. SPERM PELLET SUSPENSION:

1. Add 100 µl KMT to each tube.
   A. If there is more than one tube per sex, add 50 µl KMT to each tube.
2. Suspend samples by very gentle aspirating and releasing fluid close to the pellet.
   A. Use care not to cause foaming of the sperm pellet.
   B. If foaming occurs, the mixing is too aggressive and more care needs to be taken in the mixing method.
3. If there are multiple tubes per sex, pool the sperm pellets into a single 50-ml Falcon tube using a disposable transfer pipett.
   A. Remove a single sperm pellet using the transfer pipette from one tube at a time and depositing that sperm pellet into a common 50-ml Falcon tube.
   B. Return the 50-ml Falcon tube that contained the sperm pellet to a tube rack.
   C. After all sperm pellets have been removed and pooled into a common 50-ml Falcon tube, aspirate the residual sperm pellet from each tube using the disposable transfer pipette and place the aspirated volume to the pooled sperm pellets.
      a. There is considerable sperm pellet residual remaining in the 50-ml Falcon tubes after a single aspiration. Use patience and a caring attitude to recover as many of the sorted sperm as possible.
      b. Likewise, before discarding the transfer pipette, allow any residual volume contained in the transfer pipette to be expelled. Allowing a little time for the residual volume to gather for expulsion is very important for maximum sperm recovery.

X. PREPARATION OF HEMACYTOMETER DILUTION TUBE:

1. Prepare a 60X dilution in a 6 ml Falcon tube.
   A. Deposit 590 µl of Formalin solution to each 6 ml Falcon tube.

B. Gently vortex each pooled sorted sperm suspension and immediately remove 10 µl from the sperm suspension using a 20 µl Pipettor.
C. Dry outside of pipet tip using a Kimwipe before depositing formalin volume into 6 ml Falcon tube.
D. Deposit semen into the formalin solution.
E. Rinse semen from the pipette by aspirating and dispensing semen into formalin solution a minimum of 4 times.

2. This represents a 1:59 dilution with a **dilution factor of (60)**.

XI. HEMACYTOMETER DILUTION PREPARATION AND COUNTING:

1. Prepare (2) hemacytometers for evaluation (4 chambers).
2. Vortex the samples prepared for hemacytometer evaluation for 30 seconds.
3. Fill all hemacytometer chambers (4) with a single draw from the hemacytometer sample tube.
4. Determine sperm counts after allowing the prepared hemacytometers to settle for a minimum of 10 minutes.
   A. **Do not allow samples to dry out**.
   B. Place hemacytometers in a high humidity chamber to allow the sperm to settle.
5. Count all the sperm contained in 25 large squares.
6. Multiply the (average sperm count) x (60 = dilution factor) x (10,000) = sperm/ml.
7. Multiply the (sperm/ml) x (volume in the 50-ml Falcon tube) = total number of sperm contained in the 50-ml Falcon tube.

XII. VOLUME ASSESSMENT:

1. Weigh the 50-ml Falcon tube to determine sorted volume.
   A. Subtract the empty tube and cap weight from the filled tube and cap to calculate the sorted sample weight.
   B. Divide the sample weight by 1.04 to determine the sorted sample volume.
2. Volume can also be calculated using the **Saratoga** spreadsheet.
   A. Simply enter the empty 50-ml Falcon tube weight, followed by the filled 50-ml Falcon tube weight.
   B. The spreadsheet will calculate the sorted volume as well as the volume of KMT extender to add to each sorted sample.

XIII. FINAL EXTENSION:

1. **Enter the total number of sperm counted per hemacytometer chamber onto the *Saratoga* spreadsheet for dose calculation.**
2. The ***Saratoga*** spreadsheet will calculate the required additional KMT extender for 66.66 x 10^6/ml final sperm concentration.
3. Add additional KMT extender as calculated for a final sperm concentration of 66.66 x 10^6/ml.

XIV. INSEMINATION PREPARATION:

1. Remove 300 µl of sperm suspension to be used for insemination.
2. Place in a room temperature (22°C) 5-mL falcon tube.
3. Transport tube (in pants pocket) to surgery room for hysteroscopic or rectally –guided insemination.

XV. INSEMINATION:

1. Using hysteroscopic or rectally-guided insemination, carefully deposit the sample onto the uterotubal papilla of the horn ipsilateral to impending ovulation.

* More details will follow regarding insemination methods.

Outline Protocol With Timeline

11:00am	A.	Collect stallion(s)
	B.	Evaluate sperm for volume, concentration, and motility
	C.	Extend ejaculate in CatKMT (24 U/ml BL catalase) to 25 x 10^6 sperm/ml
	D.	Store samples for 18h
	E.	Divide the bags according to the treatment groups
		a. Treatment A&B: 5° storage in an equitainer
		b. Treatment C: 15° storage in a water bath in cold room
6:00am	F.	Remove all samples from controlled temperatures
	G.	Place samples in a water bath at ambient temperature for 30 minutes
6:30am	H.	Allow 15 min for equilibration once samples have reached room temperature
6:45am	I.	Unpackage samples and transfer to 50-mL weighed tubes
6:55am	J.	Place 50-mL tubes in centrifuge, 600g, 10 min
	K.	Evaluate post-shipping motility
7:10am	L.	Remove samples from centrifuge and aspirate samples to ~1.5ml
	M.	Gently resuspend each pellet with transfer pipettes (pellets are very soft)
	N.	Pool pellets from similar treatments
7:15am	O.	Prepare hemacytometers for each shipping treatment (2)
7:40am	P.	Extend sperm samples to 400 x 10^6 sperm/mL
7:45am	Q.	Prepare staining samples
7:50am	R.	Place samples in circulating water bath at 34° C
8:05am	S.	Place KMT with food dye in water bath
8:20am	T.	Remove samples from water bath
	U.	Add KMT with food dye (warmed) to all stained samples (A)
	V.	Filter samples using yellow partec filters
	W.	Evaluate motility of stained samples and record
8:30am	X.	Give samples to flow operators for separation
10:30am	Y.	Begin post-sort processing of sperm
	Z.	Remove a portion of each treatment and begin resort analysis

[redacted]

10:30am	AA.	Centrifuge sorted sperm a. 850 x g b. 20 min.
10:55am	BB.	Aspirate samples to ~100µl
	CC.	Add 100µl KMT to each pellet
	DD.	Prepare hemacytometers
**11:05am	EE.	Evaluate 0h post-sort motility a. Evaluate motility in KMT (non-stimulated) i. 25 µl KMT: 25µl sperm b. Dilute each sample in 4mM Caffeine for stimulated motility evaluation i. 25µl KMT with 4mM Caffeine: 25µl sperm c. Evaluate and record motility after 5-10 min. incubation in warm block
11:15am	FF.	Count hemacytometers
	GG.	Extend sperm to 66.66 x 10^6 sperm/ml for insemination
	HH.	Prepare 300 µl for immediate insemination

** Stallions for following day need to be collected at 11:00 each morning

Summary, [redacted] [redacted]

Summary of Equine Trials

[redacted]

Several in vitro studies have been performed this year in an attempt to improve viability and staining of stallion sperm for flow-sorting. To date, they have focused on shipping media, shipping temperature, sperm concentration during staining, stain concentration during staining, pH during staining, osmolality during staining, length of staining, and sperm assay methods. Much has been accomplished from the results of these trials regarding improvements to sperm viability. This report is an attempt to summarize the work that has been completed up to this point.

Experiment #1

Determination of the preferred shipping media for stallion sperm destined for flow-sorting.

Scope:

8 stallions used
4 media used – EZ Mixin CST w/Ticarcillin (20°C), KMT (20°C), Next Generation (20°C), and INRA 96(15°C)
2 pH levels at staining – 7.1 and 7.5 – This data was discarded do to unknown fluctuations in media pH.

We learned:

Immediately following shipping, motility in all media were similar
Following centrifugation and following staining, sperm processed in KMT exhibited the highest percentage of motile sperm, and KMT and INRA96 were both preferred over EZ Mixin and Next Generation.
Following high dilution and recentrifugation, sperm in INRA96 exhibited the highest percentage of motile sperm, followed by KMT, and then EZ Mixin.

Preliminary work for Exp. 1

Stallion selection – Morphology, prior success/failure on flow trials, sperm numbers produced, and availability were used to determine which stallions would be used for Exp. 1.

Recovery rates for sperm post-centrifugation – After shipment, sperm were to be centrifuged at 600g for 10 min. (instead of 450g for 15). Recovery rates with this new protocol were

evaluated and were similar to previous recovery rates (~85%, highly dependant on stallion).

PH adjustment – pH of media was adjusted to allow for 7.1 and 7.5 during staining. Media was also adjusted to bring the pH of samples back to 7.1 following staining.

Flow assessment following high dilution – A protocol was needed to determine the viability of sperm following high dilution and centrifugation using the flow. Work was done with bull sperm in which various levels of red food coloring was added back following centrifugation. These samples were run, and those with addition of media at 1:1 staining talp to pink talp allowed for separation of a dead population. This was repeated with stallion sperm, and was satisfactory. Unfortunately, this was not a reliable assay for the study, as motility did not correlate at all with percent dead readings. Apparently, live sperm had lost staining during high dilution, and fell out of the stained region along with dead sperm. This is an area that requires further investigation.

Track motilities – Track motility analysis was attempted with stallion sperm. Due to the milk necessary in stallion media, too much background signal was present. This is not a reliable assay at this time.

Experiment #2a

Optimization of staining –The effects of sperm concentration and seminal plasma during staining.

Scope:

3 Stallions – preliminary trial
2 Seminal plasma concentrations during staining – 0%, 10%
4 Sperm/stain concentrations – 50 x 10^6 /mL, 2.6µl HO; 50 x 10^6 /mL, 3.9µl HO; 150 x 10^6 /mL, 7.8µl HO; 450 x 10^6 /mL, 23.4µl HO
2 Extenders – EZ Mixin, KMT
2 processing times – Immediately after collection, after 18h storage at room temp.

We learned:

Shipped sperm exhibit better resolution, lower motility, and higher percent dead than fresh sperm.
Sperm stored and processed in KMT exhibit higher motility and better resolution than sperm stored and processed in EZ Mixin.
Seminal plasma during staining has no effect on motility or resolution. Sperm stained in 10% seminal plasma contain a higher percentage of dead sperm.
As sperm concentration during staining decreases, motility increases and resolution fades.
Shipped sperm are affected by changes in sperm concentration to a greater degree than are fresh sperm.

Experiment #2b

Optimization of staining – The effects of pH and osmolality during staining.

Scope:

2 stallions – Only one could be used
2 osmolalities during staining – 310, 360
3 levels of pH during staining – 7.1, 7.5, 7.9

***Trends:**

Staining at a higher pH tends to improve resolution.
The effects of osmolality could not be determined.

* Not reliable results because only 1 stallion was used

Experiment #2c

Optimization of staining/sperm evaluation – The effects of pH at staining, length of staining, and the amount of red food coloring added; Evaluation of caffeine and NaPyruvate as motility stimulants.

Scope:

3 stallions – preliminary trial
2 pH levels – 7.1, 7.9
2 lengths of staining
4 levels of red food dye added after staining – 1.25µl/ml 5%, 1.00µl/ml 5%, 0.75µl/ml 5%, 1.5µl/ml 2%
2 motility stimulants

We learned:

Samples stained at pH 7.9 contained lower percent dead and exhibited better resolution than those stained at pH 7.1.
Samples stained for 30 minutes contained lower percent dead and exhibited better resolution than those stained for 60 minutes.
A final red dye concentration of 0.75µl/ml produced the desired results – There is a separate dead population that is more ideally centered on the histogram.
Caffeine is capable of stimulating motility of stallion sperm at a concentration of 2mM, while NaPyruvate has no stimulatory effect on motility.

Summary, [redacted] [redacted]

Experiment #2d

Verification that the staining protocol used in 2000 is capable of producing adequate resolution and a comparison of 2 shipping temperatures.

Scope:

3 stallions – preliminary trial 4 1 1 1

5 Hoechst concentrations – 8.4µl/ml, 10.4µl/ml, 12.4µl/ml, 14.4µl/ml, 16.4µl/ml

2 shipping temperatures – 15°C, 20°C

We learned:

Shipping sperm at 15°C maintains more viable sperm than shipping at 20°C.

Resolution appears to be similar or better than last year with a similar protocol.

Resolution appears best when stained with 12.4µl/ml HO (200 x 10^6 sperm/ml)

Experiment #3

Identification of the preferred shipping temperature for stallion sperm prior to sex-selection.

Scope:

8 stallions

5 shipping temperatures – 5°C, 10°C, 15°C, 20°C, 25°C

2 versions of KMT – original (as in previous XY trials), and modified

We learned:

Sperm stored at 15°C contained the lowest percent dead, highest post-ship motility, and highest post-dilution motility when stimulated.

Sperm stored at 5°C exhibited the highest motility post-stain and post-dilution, when sperm were not stimulated.

Sperm stored at 25°C contained the highest percent dead, exhibited the lowest motility at all readings, and exhibited the poorest resolution.

Sperm stored in modified KMT maintained higher motility than those stored in original KMT.

Motility results based on non-stimulated vs stimulated evaluations differed post-stain and post-high dilution.

Storage of sperm at 5°C looks like a possible alternative to 15°C, but further studies involving evaluation after sorting are needed.

Experiment #4

The effects of sperm concentration, Hoechst concentration, and length of staining on sperm viability and resolution.

Scope:

8 stallions
2 sperm concentrations during staining – 100 x 10^6/ml, 200 x 10^6/ml
3 stain concentrations – 100% (12.4µl/200 x 10^6 sperm), 85%, 70%
2 lengths of staining – 30 min, 60 min

We learned:

Samples stained at 100 x 10^6 sperm/ml contained a lower percent dead sperm than samples stained at 200 x 10^6 sperm/ml and produced similar resolution.
As stain concentration increases, percent dead increases as well.
Sperm stained at 85% stain concentration produced similar resolution as those stained at 100%.
Staining length had no effect on either percent dead or resolution.

Effect of Stallion

	Pship	Pcent	Pstain	Hdil	% dead	resolution
A	63.3	52.5	44.5	47.5	22.8	5.7
B	54.5	50.8	32.3	31.8	41.1	8.2
C	68.8	62.8	55.6	44.1	21.4	6.2
D	64.7	58.4	46.6	48.4	26.1	7.3
E	65.3	57.8	48.8	45.5	21.6	5.2
G	58.8	58	36.8	37	30.7	7
H	58	51.5	48.5	46.5	25.2	8
J	58	50	41.8	41.8	17.9	6.8

Effect of Extender

	Pship	Pcent	Pstain	Hdil	% Dead	Resolution
KMT	60.7	52.8	42.8	40.9	26.6	6.8
KMT mod	61.6	57.1	45.2	44.4	25.5	6.8

Effect of Shipping Temperature

	Pship	Pcent	Pstain	Hdil	%Dead	Resolution
5	62.5	56.1	48.8	45	27.9	6.9
10	62	58	47.5	44.7	26.4	6.8
15	64.7	56.3	44.5	44.8	23.1	6.7
20	59.2	53.6	42.2	41.4	23.3	6.6
25	55.8	49.4	34.6	35.4	31.4	7.2

Post-Ship Motility

	Total	Prog.	Stim., T	Stim., Prog.
5	62.5	59.2	62.5	61.4
10	62	58.9	63.3	61.9
15	64.7	62.7	64.2	62.7
20	59.2	59.1	62.3	61.1
25	55.8	55	59	57.9

Post-Centrifugation Motility

	Total	Prog.	Stim., T	Stim., Prog.
5	56.1	55.6	56.3	55.2
10	58	56.3	56.4	54.4
15	56.3	56.1	53.6	52.2
20	53.6	52.8	56.4	55.6
25	49.4	48.5	52.7	51.9

Post-Staining Motility

	Total	Prog.	Stim., T	Stim., Prog.
5	48.8	48.6	49.1	49.1
10	47.5	47.2	52	51.9
15	44.5	44.5	51.4	51.3
20	42.2	42.2	48.1	47.5
25	34.6	34	42.7	42.7

Post-High Dilution Motility

	Total	Prog.	Stim., T	Stim., Prog.
5	45	45	49.5	49.5
10	44.7	44.1	49.5	48.9
15	44.8	44.8	51.1	51.1
20	41.4	40.5	46.9	46.9
25	35.4	36.9	44.4	43.3

Percent Dead and Resolution

	% Dead	Resolution
5	27.9	6.9
10	26.4	6.8
15	23.1	6.7
20	23.3	6.6
25	31.4	7.2

KMT vs mod, Post-ship motility

	KMT	mod
5	62.8	62.2
10	61.9	62.2
15	63.4	65.9
20	57.8	60.6
25	56.3	55.4

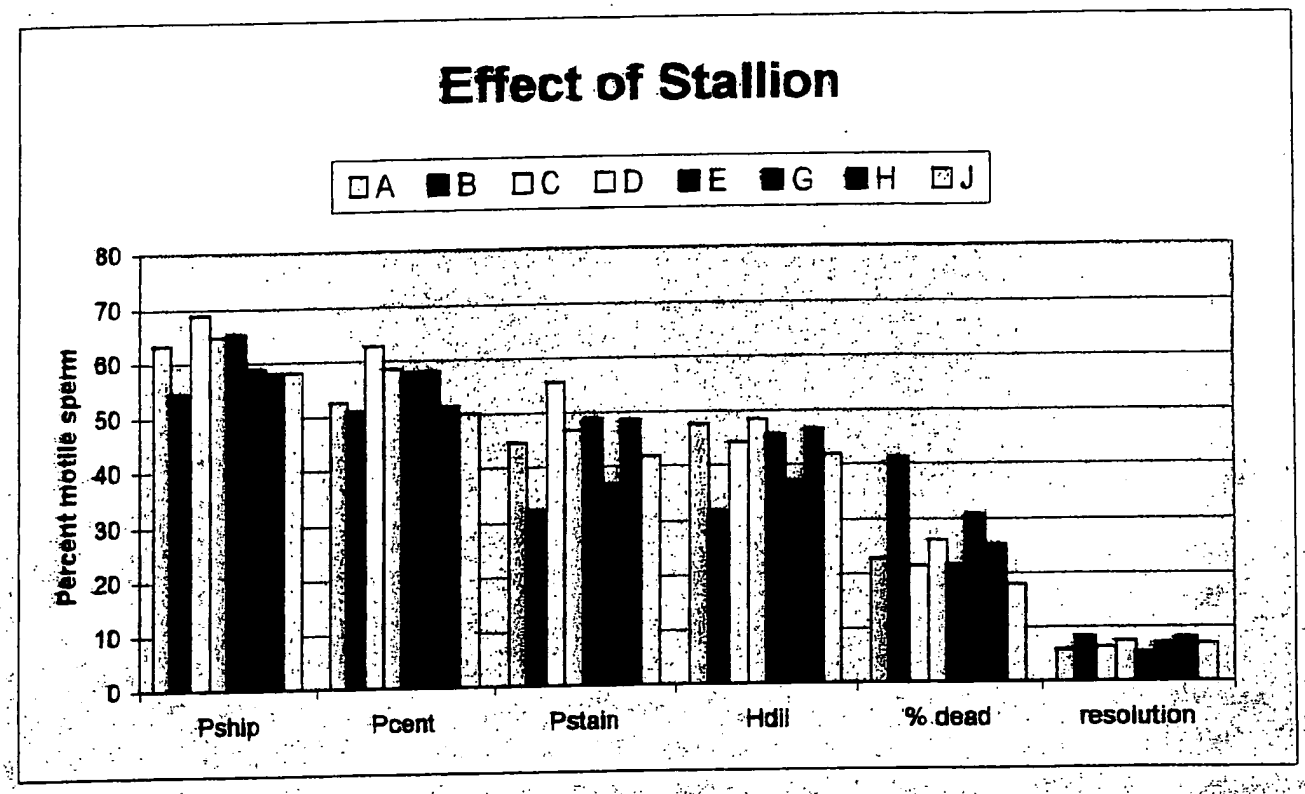
KMT vs mod, High Dilution motility

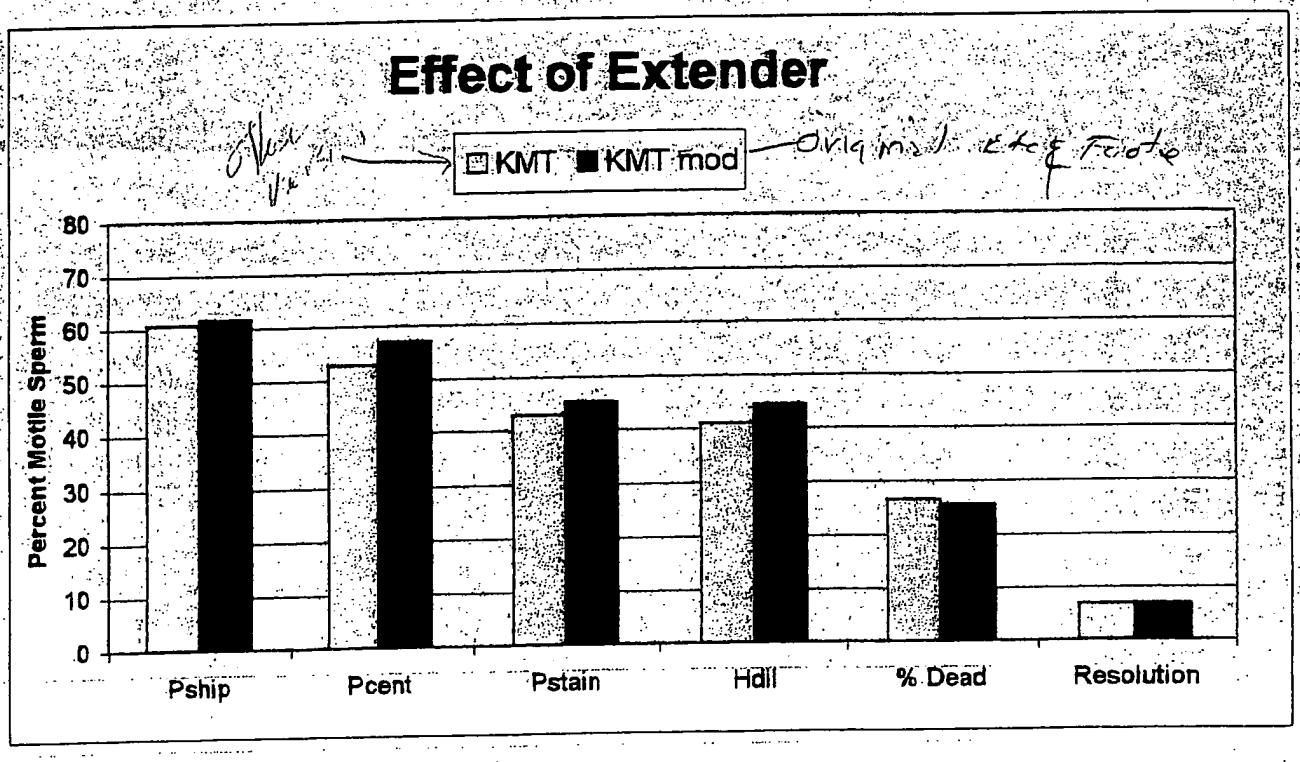
	KMT	mod
5	42.8	47.2
10	44.4	45
15	43.1	46.6
20	40	42.8
25	31.7	39.2

KMT vs mod, Percent dead

	KMT	mod
5	27.5	28.4
10	26.4	26.5
15	23.3	23
20	23	23.6
25	36.8	26

Exp. 3, Temperature x KMT





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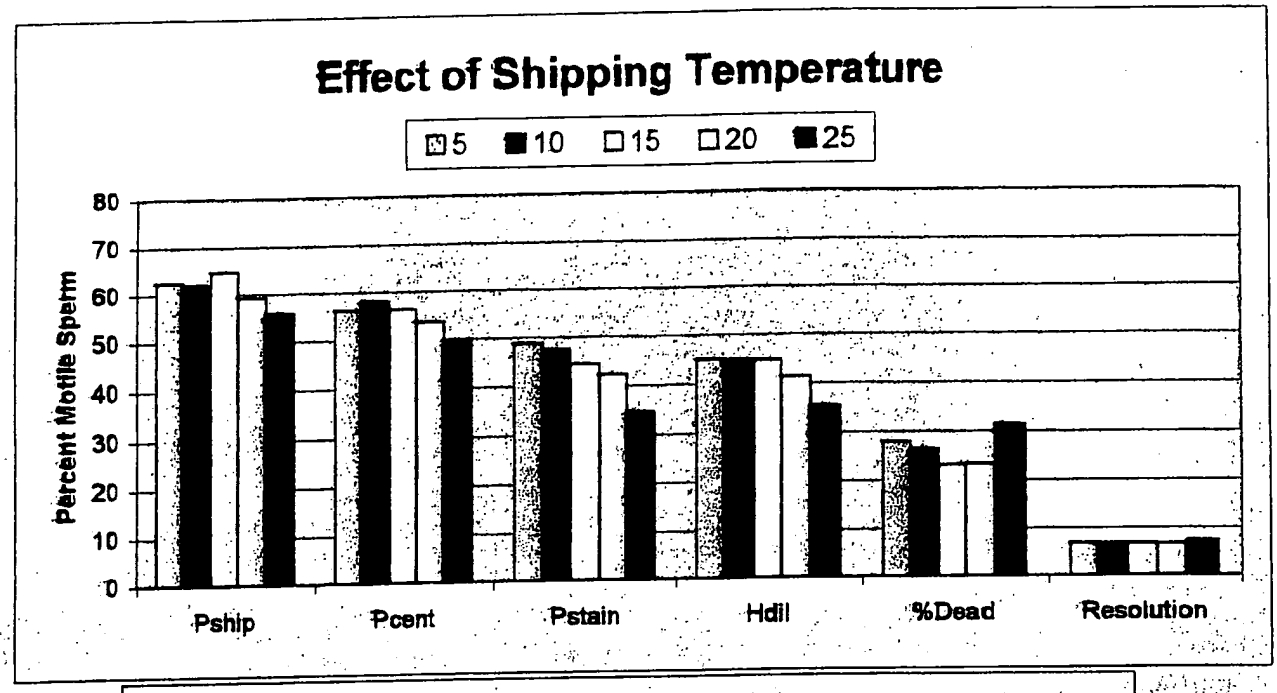

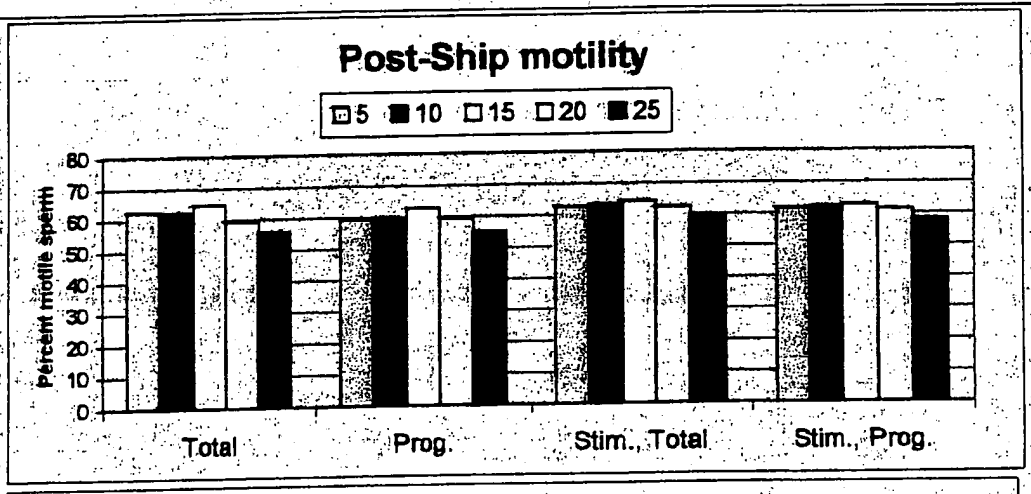

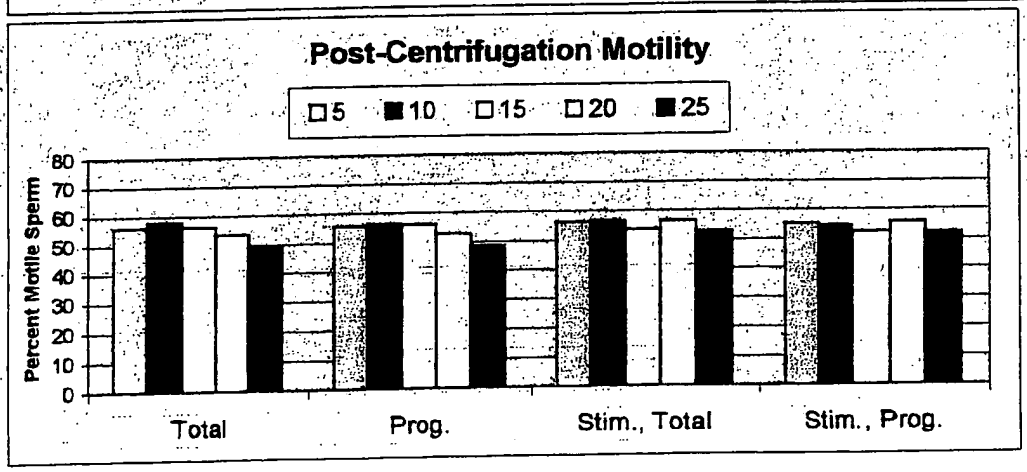

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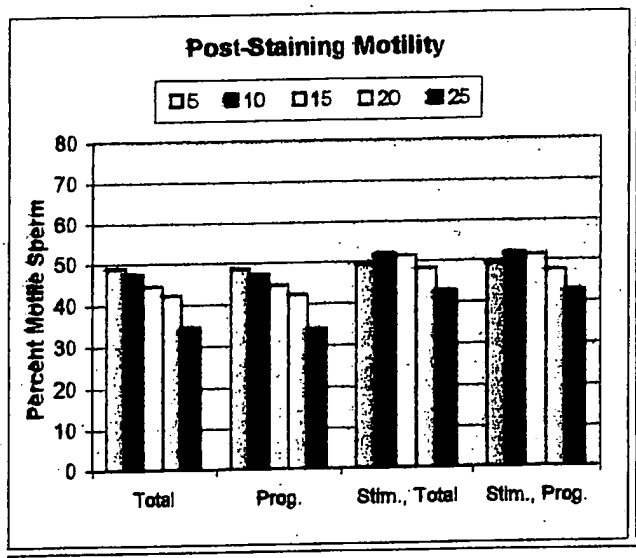

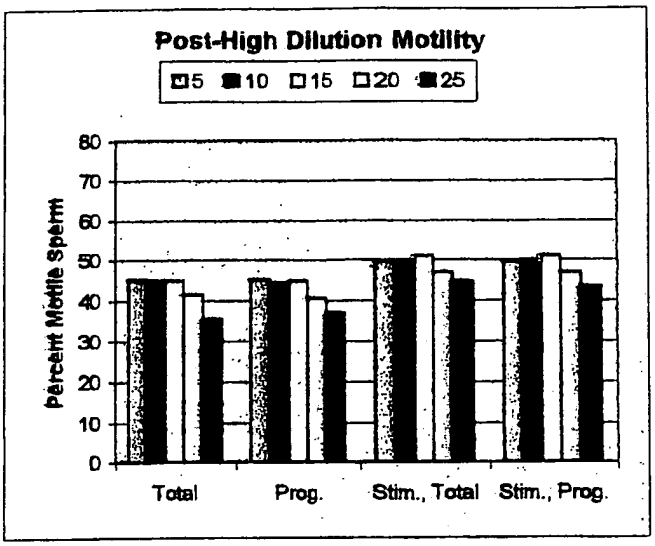

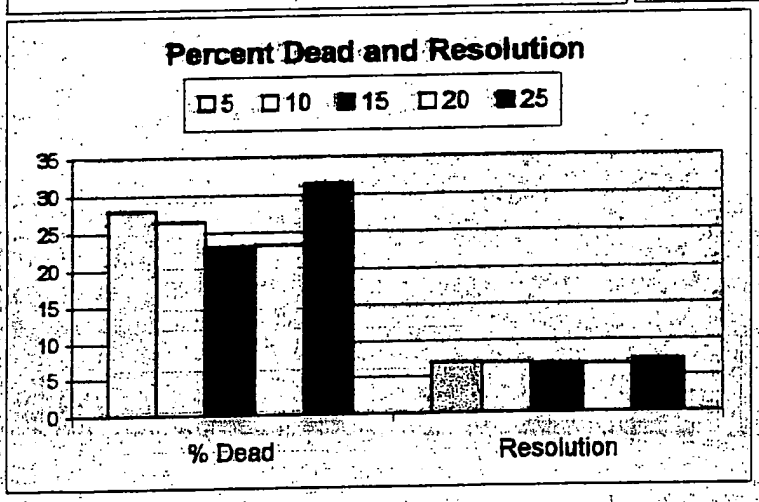

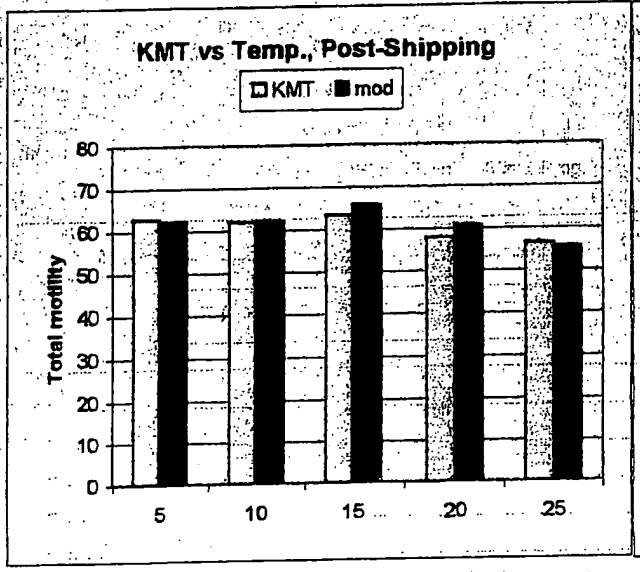

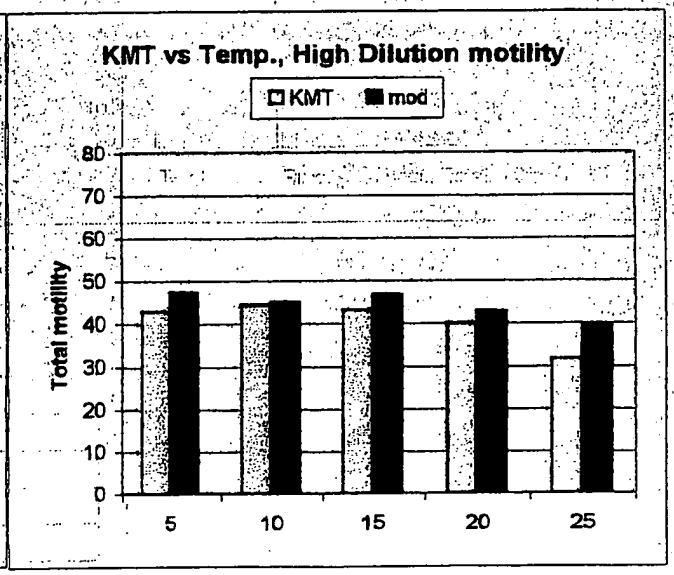

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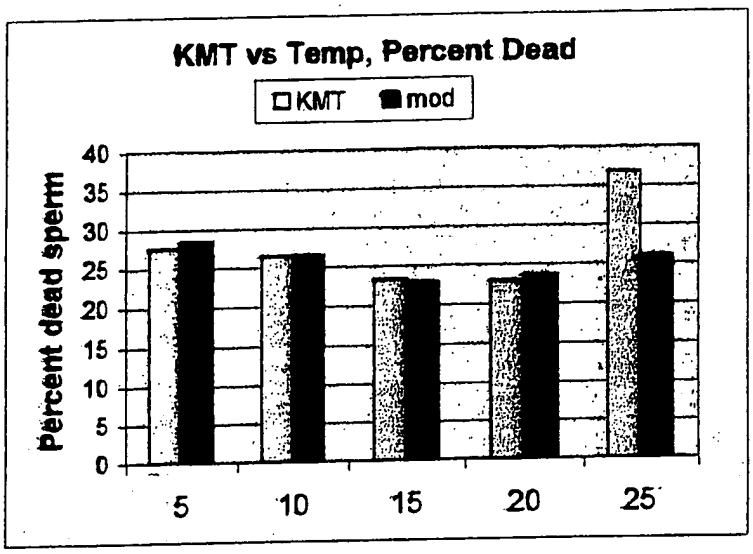
Exp. 3, Temperature x KMT



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Re: EVJ 01/33
EVJ 01/34

Pages: 20 (incl. cover)

Please find to follow the corrected proofs of two articles which are due to be published in a forthcoming issue of Equine Veterinary Journal:

EVJ 01/33: Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa

EVJ 01/34: Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa

For each article, I have sent the corrected proof, a list of author corrections, and a reprint request form. Please feel free to contact me with any additional questions.

Kind regards,

Allison Lindsey
Equine Specialist, XY, Inc.

FAXED [redacted]
BY:

EXHIBIT H

EQUINE VETERINARY JOURNAL
Equine vet. J. [redacted]

Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa

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Keywords: horse; low dose insemination; sexed semen; frozen/thawed semen; equine

Summary

The objective of this experiment was to determine the effects of flow cytometric sorting and freezing on stallion sperm fertility. A 2 x 2 factorial design was used to delineate effects of flow sorting and freezing spermatozoa. Oestrus was synchronised (July-August) in 41 mares by administering 10 ml altrenogest (2.2 mg/ml) *per os* for 10 consecutive days, followed by 250 μg cloprostenol i.m. on Day 11. Ovulation was induced by administering 3000 iu hCG i.v. either 6 h (fresh spermatozoa) or 30 h (frozen/thawed spermatozoa) prior to insemination. Mares were assigned randomly to one of 4 sperm treatment groups. Semen was collected from 2 stallions with an artificial vagina and processed for each treatment. *Treatment 1* (n = 10 mare cycles) consisted of fresh, nonsorted spermatozoa and *Treatment 2* (n = 16 mare cycles) of fresh, flow sorted spermatozoa. Spermatozoa to be sorted were stained with Hoechst 33342 and sorted into X- and Y-chromosome-bearing populations based on DNA content using an SX MoFlo sperm sorter. *Treatment 3* (n = 16 mare cycles) consisted of frozen/thawed nonsorted spermatozoa (frozen at 333 x 10^6 sperm/ml in 0.25 ml straws) and *Treatment 4* (n = 15 mare cycles) of flow sorted frozen/thawed spermatozoa (frozen at 64.4 x 10^6 sperm/ml). Concentrations of sperm in both cryopreserved treatments were adjusted, based on predetermined average post-thaw motilities, so that each insemination contained approximately 5 x 10^6 motile spermatozoa. Hysteroscopic insemination of 5 x 10^6 motile spermatozoa in a volume of 230 μl was used for all treatments. Pregnancy was determined ultrasonographically 16 days postovulation.

No differences were found (P>0.1) in the pregnancy rates for mares inseminated with fresh nonsorted (4/10 = 40.0%), fresh flow sorted (6/16 = 37.5%), frozen/thawed nonsorted (6/16 = 37.5%) and flow sorted frozen/thawed spermatozoa (2/15 = 13.3%). Pregnancy rates tended (P = 0.12) to be lower following insemination of frozen/thawed flow sorted spermatozoa. Further studies are needed with a larger number of mares to determine if fertility of flow sorted frozen/thawed spermatozoa can be improved.

Introduction

A safe and reliable method for preconceptual sex selection of offspring has been sought for decades in man, livestock and companion animals. Johnson *et al.* (1989) were the first to report a reliable method to predetermine sex, by using DNA as a quantitative marker for X- and Y-chromosome-bearing spermatozoa and sorting spermatozoa via flow cytometry. This method was used subsequently to sort stallion spermatozoa and produce foals of predetermined sex (Buchanan *et al.* 2000; Schmid *et al.* 2000), but additional studies are required to make this technique applicable in the horse industry. Flow sorted stallion spermatozoa will be of limited use until methods for inseminating low numbers of spermatozoa are improved and until a successful cryopreservation technique is developed for flow sorted stallion spermatozoa.

Pickett and Voss (1975) determined that maximum fertility in mares is obtained by using a single insemination dose of 500 x 10^6 progressively motile spermatozoa (pms). At current rates at which stallion sperm can be sorted by flow cytometry, more than 5 days of continuous sorting would be required to accumulate 500 x 10^6 spermatozoa of each sex. Recently, several new techniques have been developed to increase fertility when using low numbers of stallion sperm. McCue *et al.* (2000) achieved a 21% pregnancy rate by surgically depositing 50,000 pms directly into the oviduct of pre-ovulatory mares. Manning *et al.* (1998) reported a 10% pregnancy rate when as few as 1 x 10^6 spermatozoa were deposited into the mare's oviduct through the uterotubal junction using a hysteroscopic technique. In a subsequent trial, a 30% pregnancy rate was reported from the insemination of 3.8 x 10^6 progressively motile spermatozoa placed on the uterotubal papilla with the use of an endoscope (Vazquez *et al.* 1998). Utilising a less invasive technique, Buchanan *et al.* (2000) inseminated 5 x 10^6 pms by use of an ultrasound guided method to direct a flexible insemination pipette to the tip of the uterine horn ipsilateral to the ovary containing the dominant follicle. The 35% pregnancy rate obtained was similar to that reported by Vazquez *et al.* (1998). Morris *et al.* (2000) used a videoendoscopic technique similar to that used by Brecher and Allen (1992) and achieved a 64% pregnancy rate when only 1 x 10^6 pms were placed onto and around the uterotubal papillae. Since this technique resulted in the highest reported pregnancy rates using low sperm numbers, we utilised videoendoscopic insemination in the present study to inseminate low numbers of flow sorted and nonsorted, fresh and frozen/thawed stallion spermatozoa.

Limited research has been reported on the fertility of flow sorted stallion sperm. The first pregnancy from flow sorted stallion spermatozoa was produced by surgical oviductal insemination (Schmid *et al.* 2000). The following season, Buchanan *et al.* (2000) reported a 40% pregnancy rate using 25 x 10^6 flow sorted stallion

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spermatozoa deposited deep into the uterine horn.

The ability to cryopreserve sorted spermatozoa would greatly increase the practicality of using flow sorted spermatozoa in the horse industry. Stallion sperm do not survive for long periods after the sorting process. Therefore, mares must be inseminated immediately after sperm sorting. However, if the sperm could be frozen following sorting, they could be used at any future time or location.

Several concerns should be addressed in the development of a cryopreservation protocol for use with flow sorted stallion spermatozoa. When using conventional techniques to freeze stallion spermatozoa, a single medium is not available that works best for all stallions (Squires *et al.* 1999). The same can be expected when freezing flow processed stallion spermatozoa but, as a means to decrease variables in studies of flow-sorted, frozen/thawed stallion spermatozoa, the freezing medium that produces the best results for most stallions should be identified and used.

Furthermore, it has been reported that spermatozoa from individual stallions can respond quite differently to cryopreservation (Muller 1987). Variation between stallions is also apparent in the ability of their sperm to be sorted by flow cytometry into X- and Y-chromosome-bearing populations. Because of these variations, it is necessary to carefully select stallions for this process that are most likely to survive the rigours of both flow sorting and cryopreservation.

Cryopreservation of flow sorted bull spermatozoa has been studied extensively. Seidel *et al.* (1999a) reported that the pregnancy rates for heifers inseminated with flow sorted frozen/thawed spermatozoa (18/35 = 51%) were similar to that for frozen/thawed nonsorted spermatozoa (27/37 = 73%; P>0.05). Seidel *et al.* (1999b) also reported that pregnancy rates for heifers inseminated with low numbers of sorted, frozen/thawed sperm are generally within 90% of rates obtained with nonsorted, frozen/thawed sperm samples containing 7 to 20 times more spermatozoa per insemination.

In addition to developing techniques for inseminating low numbers of sperm for use with flow sorted spermatozoa, insemination with low numbers of nonsorted frozen/thawed spermatozoa is also of interest. This is particularly true for stallions with a limited inventory of frozen samples, for those that are dead or no longer capable of producing fertile spermatozoa, and for those that have poor sperm quality after cryopreservation.

The present study used a 2 x 2 factorial design to determine the effects of flow cytometric sex selection and cryopreservation of sperm on the fertilizing capacity of stallion spermatozoa. The objectives of this study were: 1) to determine if flow sorting stallion spermatozoa decreased pregnancy rates at an insemination dose of 5 x 10^6 pms; 2) to compare pregnancy rates of mares inseminated with low numbers of fresh sperm to those with frozen/thawed sperm; and 3) to determine whether pregnancies can be achieved when low numbers of flow sorted frozen/thawed stallion spermatozoa are inseminated into mares.

Materials and methods

A preliminary experiment was conducted to determine the preferred medium for cryopreservation and selection of stallions to be used in this study, as follows:

Semen collection and evaluation

Semen was collected from each of 7 stallions using a CSU model artificial vagina[1] equipped with an inline gel filter. After collection, the semen was evaluated for gel-free volume, motility and sperm concentration. The neat ejaculate was extended at a ratio of 1:1 (extender:semen, v/v) with prewarmed HBGM-3 (adapted from Parrish *et al.* 1988) and centrifuged at room temperature for 15 min at 400 *g* to concentrate the spermatozoa and decrease seminal plasma concentration. After centrifugation, the supernatant containing 50% of the seminal plasma was removed, leaving a soft sperm pellet of ~3 ml. The postcentrifugation concentration (~1200 x 10^6 sperm/ml) was determined using the Densimeter[1].

Processing of control semen

An aliquot was taken from this sample and frozen using conventional freezing methods (Squires *et al.* 1999) for stallion spermatozoa (control). Sperm were extended to a final freezing concentration of 20 x 10^6 sperm/ml in room temperature lactose EDTA extender. Extended sperm were loaded into 0.25 ml straws and placed on a freezing rack at room temperature. The loaded freezing rack was then placed in static liquid nitrogen vapour at approximately -100°C where straws were allowed 5 min to freeze, then plunged into liquid nitrogen for storage.

Processing of treated spermatozoa

The remaining sample was processed by a method which simulated preparation for flow sorting; however, the sperm were not processed through the MoFlo[2] instrument.

All treatment samples were processed identically until freezing. After centrifugation, the spermatozoa were extended to 400 x 10^6 sperm/ml in HBGM-3, in a total volume of 1 ml. Each sample was stained with 25 µL Hoechst 33342[3] (prepared in deionized water at 5 mg/ml) and incubated at 34°C for 1 h. Following incubation, samples were diluted to 100 x 10^6 sperm/ml with the addition of 3 ml prewarmed HBGM-3 containing 2 µl/ml red food colouring (1% FD&C No. 40). Samples were then filtered through a 40 micron filter apparatus into a 6 ml polypropylene tube.

Samples were prepared in triplicate (3 potential freezing extenders) with sheath fluid (HBGM-3). Sperm were diluted in sheath fluid and freezing extender without glycerol (representing catch fluid) at 600,000 sperm/ml. In a 50 ml Falcon tube, 150 µl of sperm sample was combined with 21 ml sheath fluid and 4 ml of each of 3 freezing extenders (lactose EDTA, FR5 and CO5; Squires *et al.* 1999). Samples were incubated for 2 h at ambient temperature to simulate the amount of time that would be needed for sorting of X- and Y-chromosome-bearing populations. Samples were then reconcentrated for freezing. Tubes were centrifuged (22°C) for 20 min at 850 *g* and the supernatant was aspirated, leaving a 200 µl sperm pellet. Based on 85% recovery rates, each pellet contained 12.75 x 10^6 sperm. To reach the desired freezing concentration of 20 x 10^6 sperm/ml, 0.44 ml of the appropriate freezing extender (with glycerol) was added to each pellet. Spermatozoa were then frozen (~8 h postcollection) according to the appropriate protocol for each extender (Squires *et al.* 1999).

Freezing of treated spermatozoa

For samples in FR5 and CO5, the 50 ml tubes containing the extended pellets were suspended in a 600 ml beaker of 22°C water, covered, placed in a 5°C cold room and allowed to cool for

90 min (-2°C/min). Samples were then gently vortexed to redistribute spermatozoa in the medium, and 0.23 ml was loaded in each precooled 0.25 ml straw. Loaded straws were placed on a precooled freezing rack, which was then placed in static liquid nitrogen vapour at approximately -100°C. After 5 min in vapour, straws were plunged into liquid nitrogen for storage.

For samples in lactose EDTA, sperm were loaded at ambient temperature (22°C) in 0.25 ml straws and frozen in a similar way to control samples.

Thawing and evaluation

To thaw sperm, straws were placed in a warm water bath (37°C) for 30 s. The end of the straw containing the stainless steel ball was cut and sperm expelled into prewarmed 1.5 ml polypropylene epitubes. Two straws/ejaculate/treatment were evaluated.

Sperm quality was determined based on post-thaw motility. Visual motilities for all samples were read by 2 technicians at 0.5 and 2 h post-thaw. Each sample was evaluated for total and progressive motility by each technician, and readings were averaged between technicians.

Treatment differences ($P<0.05$) with respect to total and progressive motility at 0.5 and 2 h were detected using Analysis of Variance in SAS. Treatment means were separated using Tukey's Studentised Range (HSD) test in the General Linear Models Procedure.

The results from this preliminary experiment were examined, and 2 stallions whose sperm exhibited acceptable post-thaw motility (>35% pms) were selected for use in the next experiment. Additionally, FR5 was chosen as the preferred freezing extender for use in this experiment, since the post-thaw motilities were generally greater for spermatozoa frozen in this extender.

Semen collection and processing

Semen was collected from each of two 4-year-old Arabian stallions on alternating days throughout the duration of the project using a CSU model artificial vagina equipped with an in-line gel filter. After collection, the gel-free volume, motility and spermatozoal concentration for each ejaculate was determined. The semen was then extended 10:1 (extender:semen) with prewarmed HBGM-3 (adapted from Parrish *et al.* 1988) and centrifuged immediately at ambient temperature for 15 min at 400 *g* to concentrate the spermatozoa and remove 90% of the seminal plasma. After centrifugation, the supernatant was removed, leaving soft sperm pellets with sperm concentrations of >1.2 x 10^9/ml. The pellets were transported immediately to another laboratory (~5 min) for further processing in one of 4 treatment groups.

Treatment 1: Mares (n = 10 cycles) were inseminated 6 h post-hCG administration with 5 x 10^6 fresh, nonsorted pms via hysteroscopic insemination. Following centrifugation, sperm were incubated in the dark in HBGM-3 at ambient temperature and in a concentration of ~1.2 x 10^9 sperm/ml for approximately 6 h (to simulate the time needed to sort spermatozoa for *Treatments 2* and *4*).

Immediately prior to insemination, motility was visually evaluated, concentration was determined using a haemacytometer, and a 230 µl dose containing 5 x 10^6 pms was prepared in a skim milk + egg yolk extender (FR4) and inseminated immediately.

Treatment 2: Mares (n = 16 cycles) were inseminated 6 h post-hCG administration with fresh, flow sorted spermatozoa via hysteroscopic insemination. The concentration of sperm in the soft pellet was determined with the Densimeter, and a volume of HEPES BGM-3 was added to bring the spermatozoal concentration to 400 x 10^6 sperm/ml. A stock solution of 8.89 mmol/l Hoechst 33342, a stain that binds to adenine-thymine-rich regions of the minor groove of the DNA helix, was prepared in nanopure water (Johnson *et al.* 1989). One ml sperm suspension was stained with 25 µl Hoechst 33342 and incubated at 34°C for 1 h. The stained samples were then diluted to 100 x 10^6 sperm/ml for sorting with the addition of 3 ml HEPES BGM-3 containing red food colouring (2 µl/ml of 1% FD&C No. 40). The samples were filtered at unit gravity through a 40 µ nylon mesh filter into 6 ml polypropylene tubes to remove any debris and clumped spermatozoa and held at ambient temperature until they were analysed and sorted.

Spermatozoa were sorted using 2 Cytomation SX MoFlo flow cytometer/cell sorters modified for sperm sorting. Argon lasers, emitting 150 mW at wavelengths of 351 and 364 nm, were used on each of 2 MoFlo instruments at 50 psi. HEPES BGM-3 prepared without BSA was used as the sheath fluid (pH = 7.2, 290–310 mOsm). Approximately 1,000 live sperm/s were sorted and collected into 50 ml centrifuge tubes containing 4 ml FR4. Tubes containing spermatozoa of corresponding sex were pooled from each flow cytometer, and sorted spermatozoa were centrifuged for 20 min at 850 *g* at 22°C. The supernatant was removed, leaving a pellet of approximately 100 µl, and the pellets then resuspended in 100 µl room temperature FR4 and gently mixed. The spermatozoal concentration was then determined using haemacytometer counts (n = 4) and the percentage of motile sperm in the sorted samples (X and Y) was evaluated visually. Additional FR4 was then added to each sample to obtain the desired final spermatozoal concentration of 21.7 x 10^6 pms/ml. The predetermined volume (230 µl) containing 5 x 10^6 motile spermatozoa was then loaded into an equine GIFT catheter[5] and inseminated using the hysteroscopic insemination technique (Morris *et al.* 2000).

Treatment 3: Mares (n = 16 cycles) were inseminated 30 h post-hCG administration with 5 x 10^6 nonsorted motile frozen/thawed spermatozoa via hysteroscopic insemination. After initial processing, the postcentrifugation concentration was determined using the Densimeter, and pellets were adjusted to their final concentration of 33.5 x 10^6 sperm/ml in a skim milk + egg yolk + 4% glycerol extender (FR5). Sperm suspensions were protected from light and held at room temperature until samples from *Treatment 4* were ready to freeze. Sealed tubes containing the sperm pellets were suspended in 600 ml beakers containing room temperature water, covered and placed in a 5°C cold room, where sperm pellets were allowed to cool slowly (~0.2°C/min) to 5°C. After 90 min, sperm were packaged into 0.25 ml polyvinlychloride straws, set on a rack and frozen in static liquid nitrogen vapour.

The contents of each straw comprised one insemination dose of 230 µl containing approximately 5 x 10^6 motile spermatozoa (average post-thaw motility = 65%). Each straw was thawed in a 37°C water bath for 30 s, the contents transferred to a prewarmed 6 ml falcon tube, and then drawn into an equine GIFT catheter for hysteroscopic insemination (Morris *et al.* 2000).

Treatment 4: Mares (n = 16 cycles) were inseminated 30 h post-hCG administration with 5 x 10^6 motile sperm that had been flow sorted

TABLE 1: Percentage of motile spermatozoa observed post-thaw of control and flow processed stallion spermatozoa frozen in 3 extenders

Extender	Total motility (0.5 h)	Progressive motility (0.5 h)	Total motility (2 h)	Progressive motility (2 h)
Control (lactose EDTA)	38[a]	34[a]	24[a]	20[a]
FR 5	28[b]	22[b]	19[b]	14[b]
Lactose EDTA	22[c]	18[b]	14[c]	8[c]
CD 5	22[c]	12[c]	7[d]	4[d]
s.e.m.*	1.53	1.53	1.06	0.86

[a,b,c,d]Values within columns with similar superscripts do not differ ($P>0.1$); * s.e.m. was calculated by taking the square root of (error term of ANOVA/n).

and then frozen and thawed. Sperm pellets for this treatment were treated identically to those in *Treatment 2* throughout the sorting process. Spermatozoa were sorted using SX MoFlo flow cytometer/sperm sorters and collected in 50 ml centrifuge tubes containing 4 ml FR4. Tubes containing spermatozoa of corresponding sex were pooled from each flow cytometer, and sorted spermatozoa were centrifuged at 22°C for 20 min at 830 *g*. The supernatant was removed, leaving a pellet of approximately 100 µl, and the pellets then resuspended in 100 µl FR5. Sealed tubes containing the sperm pellets were suspended in 600 ml beakers containing room temperature water, covered and placed in a 5°C cold room, where sperm pellets were allowed to cool slowly (~0.2°C/min) to 5°C over a 90 min period. Haemacytometer counts ($n = 4$) were performed to determine the postcentrifugation concentration, and a volume of FR5 was then added to each tube at 5°C to obtain a final concentration of 64.4×10^6 sperm/ml. Sperm samples were vortexed gently, loaded into 0.25 ml polyvinylchloride straws, and frozen as for *Treatment 3*.

The contents of each straw comprised one insemination dose of 230 µl which contained approximately 5×10^6 motile spermatozoa (average post-thaw motility = 35%). Each straw was thawed in a 37°C water bath for 30 s, the contents transferred to a 6 ml tube and then loaded into an equine GIFT catheter for immediate hysteroscopic insemination (Morris *et al.* 2000).

Mare management

Forty-one mares of light-horse type, age 3–10 years, were synchronised by administering altrenogest (Regumate)[4] (2.2 mg/ml *per os*; 10 ml/hd/day) for 10 consecutive days, followed by an injection of cloprostenol (Estrumate)[5] (250 µg, i.m.) on Day 11. After cloprostenol administration, mares were examined via rectal palpation and ultrasonography once every other day until a follicle ≥ 30 mm diameter was detected. Mares with large follicles (≥30 mm) were examined each morning until a follicle ≥35 × 35 mm was detected. These mares were immediately administered 3000 iu human chorionic gonadotropin (Chorulon)[6] (hCG) and assigned randomly to one of the 4 treatment groups. Mares were inseminated with fresh sperm 6 h post-hCG, and with frozen/thawed sperm 30 h post-hCG.

All mares were inseminated hysteroscopically (Bracher and Allen 1992; Morris *et al.* 2000) with a total volume of 230 µl. Briefly, the sperm were loaded into an equine GIFT catheter using a 6 ml disposable syringe attached to the injection port on the distal end of the catheter. The loaded catheter was drawn into an outer polypropylene cannula, which was then passed down the working channel of a Pentax pediatric EC3430F endoscope[6]. The flexible endoscope (1.6 m long with an outer diameter of 12 mm) was guided through the cervix and filtered air was introduced into the uterus to facilitate passage of the instrument through the uterine lumen. With the aid of a video monitor, inseminators directed the endoscope through the lumen of the uterine horn ipsilateral to the ovary containing the preovulatory follicle. When the tip of the endoscope came to within 3–5 cm of the papilla of the uterotubal junction, the GIFT catheter containing the sperm suspension was extruded from the working channel of the endoscope and placed against the papilla. The plunger of the syringe was then depressed, depositing the small volume of inseminate on and around the surface of the papilla. The endoscope was then withdrawn steadily from the uterus while simultaneously evacuating the filtered air from the uterine lumen.

All mares were inseminated only once, on the side ipsilateral to impending ovulation. To determine the day of ovulation, mares were examined using ultrasound daily after insemination until ovulation was detected. Pregnancy examinations were performed ultrasonographically on Days 12, 14, 16, 25 and 35 after ovulation (day of ovulation = 0). Pregnancy status was determined based upon Day 16 examination. Eight mares that became pregnant after insemination with flow sorted sperm were allowed to foal to determine the normality of offspring resulting from flow sorted spermatozoa, as well as to confirm the resulting sex of the foals.

Chi-square analysis was used to test for differences ($P<0.05$) in the fertilising ability of spermatozoa in the 4 treatments.

Results

The results of the preliminary experiment comparing various extenders for cryopreservation are presented in Table 1. Post-thaw motilities of spermatozoa frozen by the control method were higher than motilities observed for all other treatments. Based on visual estimates of the percentage of motile sperm, FR5 was the preferred extender for cryopreservation of flow processed stallion spermatozoa. Spermatozoa that were processed for flow cytometry and subsequently frozen in FR5 exhibited the greatest percentage of motile spermatozoa post-thaw at 0.5 h (28%; $P<0.05$), as well as the highest percentage of progressively motile sperm at 2 h (14%; $P<0.05$) (Table 1).

Substantial variation was detected among the stallions used in the preliminary experiment, as detected by post-thaw motilities of control and flow processed spermatozoa. Post-thaw motilities for

TABLE 2: Variation among stallions in the percentage of total and motile spermatozoa post-thaw, averaged across 3 freezing extenders

Stallion	Total motility (0.5 h)	Progressive motility (0.5 h)
A	41[a]	28[a]
B	36[b]	32[a]
C	35[c]	28[a]
D	24[b]	20[b]
E	23[b]	18[b,c]
F	17[c]	16[c,d]
G	13[c]	10[d]
s.e.m.*	2.02	2.03

[a,b,c,d]Values within columns with similar superscripts do not differ (P>0.1); * SEM was calculated by taking the square root of (error term of ANOVA / n).

the 7 stallions are shown in Table 2. Total post-thaw motilities (0.5 h) ranged from 41–13%, and progressive post-thaw motilities (0.5 h) were 32–10%. Motilities observed at 2 h post-thaw are not presented, but they followed a similar trend as did motilities read at 0.5 h. Among stallions, total motilities (2 h) ranged from 30% (Stallion B) to 7% (Stallion F), and progressive motilities (2 h) from 24% (Stallion B) to 4% (Stallion F).

In the present trial, pregnancy rates were not different between stallions (Stallion A = 9/28, 32%; Stallion B = 9/29, 31%; P>0.1); therefore, fertility data were combined. No differences were found in the pregnancy rates of mares inseminated with fresh nonsorted, fresh flow sorted, frozen nonsorted and flow sorted frozen/thawed spermatozoa (Table 3). There was a tendency for pregnancy rates to be lower following insemination of flow sorted frozen/thawed spermatozoa when compared to all other treatments (13 vs. 38%; P = 0.12).

The mean diameter of the preovulatory follicle at the time of hCG treatment was 37.1 mm, range 33.5–45.0 mm. Ovulation was detected on Day 2 after hCG treatment in 95% of the mares, range 1–4 days.

One mare that became pregnant from insemination of flow sorted, frozen/thawed spermatozoa aborted the fetus at 246 days gestation. The fetus was of the correct predetermined sex. All of the remaining mares that became pregnant with flow sorted sperm were allowed to foal and the sex of 6 of the 7 foals corresponded correctly to the spermatozoa used. Therefore, of the 8 mares impregnated with sex-selected sperm, 7 foals (88%) were of the correct predetermined sex (Table 3).

Discussion

Upon comparison of freezing extenders in the preliminary experiment, FR5 was found to be the most desirable based on post-thaw motility of flow processed stallion spermatozoa. It should be realised, however, that FR5 may not be the preferred freezing extender for all stallions. For instance, spermatozoa collected and processed from Stallion F exhibited higher (P<0.05) progressive post-thaw motility when frozen in lactose EDTA (17%), than in FR5 (4%). Prior to application of this technology for individual stallions, it is recommended that each particular stallion be evaluated in each extender as a means of optimising potential fertilising capacity.

In previous studies of frozen stallion spermatozoa, great variation among stallions regarding the freezing ability of semen has been observed repeatedly. Pickett and Amann (1993) estimated that 25–30% of stallions produce semen that cryopreserves well, 25–30% produce semen that cryopreserves moderately and 25–40% semen that cryopreserves poorly. Similar results were obtained in the present trial with flow processed spermatozoa, except post-thaw motilities were even further depressed.

For commercial application of flow sorted frozen/thawed stallion spermatozoa, total post-thaw motilities >30% are desirable. Based on the present study, 3 of 7 stallions (43%) would qualify as candidates for future flow sorting applications. Stallions A and C were chosen to be used in the present study to compare pregnancy rates of mares inseminated with low numbers of flow sorted frozen/thawed stallion spermatozoa. Additionally, due to higher post-thaw motilities exhibited by sperm frozen in FR5, this extender was chosen as the cryopreservation medium to be used in the present experiment.

The recommended insemination dose to achieve maximum fertility in mares, as suggested by Pickett and Voss (1975), remains as 500 x 10^6 pms inseminated every other day while the mare is in oestrus. However, in several studies, no decrease in fertility has been found when researchers used only 100 x 10^6 pms (Pickett *et al.* 1974; Demick *et al.* 1976), although inseminating 50 x 10^6 pms did reduce pregnancy rates (Pickett *et al.* 1976). Due to the fact that only limited numbers of spermatozoa are available following flow sorting, and many of these may have compromised function, it is imperative that insemination techniques be developed that permit low numbers of spermatozoa to be inseminated into mares without reducing fertility.

Perhaps the most exciting finding of the present experiment is the 38% pregnancy rate achieved after insemination of only 5 x 10^6 motile frozen/thawed spermatozoa. Vidament *et al.* (1997) and Loipold *et al.* (1998) estimated that approximately 300 x 10^6 pms is the optimum insemination dose for frozen/thawed stallion

Table 3: Pregnancy rates from a single insemination of nonsorted and flow sorted, fresh and frozen/thawed equine spermatozoa

Treatment	Mares inseminated	Mares pregnant day 16 (% pregnant)	Sperm inseminated ♀ ♂	Resulting sex (foal) ♀ ♂
Nonsorted fresh	10	4 (40)	-- --	-- --
flow sorted fresh	16	6 (38)	4 2	4 2
Nonsorted frozen	16	6 (38)	-- --	-- --
Flow sorted frozen	15	2 (13)	2 --	1[a] 1

[a]This mare lost her pregnancy at ~9 months; the fetus was male.

spermatozoa to be used for insemination, and pregnancy rates of 26 and 40% per cycle were achieved, respectively). The number of spermatozoa inseminated in the present study was less than 2% of this recommended minimum number of sperm, but similar pregnancy rates were achieved (38%). The success in the present study can be attributed, in part, to the use of videoendoscopic insemination, which permitted placement of the sperm directly onto the papilla of the uterotubal junction.

The significance of these results achieved using low numbers of nonsorted frozen/thawed spermatozoa should not be underestimated by the equine industry. With the ability to use only 5×10^6 motile spermatozoa per insemination, current frozen semen inventories could be used to inseminate increased numbers of mares. Stallions labelled as 'poor freezers' may also benefit from this insemination method. These benefits are more difficult to predict, however, as the cause of these 'poor freezers' is not yet fully understood.

The lack of a significant difference between the pregnancy rates obtained from the use of nonsorted vs. flow sorted spermatozoa is in agreement with previous studies in our laboratory (Buchanan *et al.* 2000). It is realised, however, that additional studies with larger numbers of mares are necessary to detect true differences in fertility of flow sorted stallion spermatozoa.

It is surprising that the pregnancy rates of flow sorted spermatozoa could equal that of nonsorted spermatozoa, since the procedure is a lengthy and highly insulting process. Prior to sorting, spermatozoa are incubated for 1 h at 34°C with Hoechst 33342. During sorting, spermatozoa are pumped at high pressure through fine tubing at ~100 km/h and are then stored for several hours diluted at 500,000 sperm/ml. Any of these steps could potentially induce harm, but it is not yet clear at which point the greatest amount of damage occurs.

For this study, the appropriate nonsorted controls were those in which spermatozoa did not undergo any of the potentially harmful treatments necessary in the sperm preparation and sorting process. Although the spermatozoa for these controls did not endure any of the necessary processing steps, the time interval from collection to insemination (or freezing) remained constant for all treatment groups. Additionally, for both frozen treatment groups, the number of motile spermatozoa to be inseminated, along with the volume of the inseminate, were the most critical factors for evaluation of the process. Therefore, due to differences in the post-thaw motilities of sorted vs. nonsorted spermatozoa, the concentrations of control and sorted sperm that were cryopreserved were not identical.

Timing of insemination in relation to hCG administration was different for fresh spermatozoa vs. frozen spermatozoa. Similar to previous work with inseminations at the uterotubal junction (Morris *et al.* 2000), hCG was administered a maximum of 8 h prior to insemination for all fresh sperm treatments (1 and 2). When using frozen/thawed spermatozoa, it has been shown that pregnancy rates are highest when insemination occurs within 12 h prior to ovulation (Amann and Pickett 1987). Therefore, spermatozoa from both frozen treatments (3 and 4) were inseminated 30 to 32 h after hCG administration. In retrospect, it might also be beneficial to inseminate fresh flow sorted spermatozoa 24 to 30 h after hCG administration so that spermatozoa were in the mare within 12 to 24 h of ovulation. In previous studies of flow sorted boar spermatozoa, it was observed that sorted sperm exhibited a higher proportion of membranes that were acrosome-reacted or precapacitated (Maxwell *et al.* 1998). Therefore, it may be that flow sorted spermatozoa are similar to frozen/thawed spermatozoa and have limited longevity in the oviduct. Pregnancy rates may be maximised, therefore, by inseminating flow sorted stallion spermatozoa within 12 h of ovulation. Timing of insemination of flow sorted stallion spermatozoa warrants additional study.

The pregnancies obtained by insemination of only 5×10^6 motile flow-sorted, frozen/thawed spermatozoa are of great interest. Although the pregnancy rates for this treatment group were low (2/15; 13%), this is the first report of pregnancies obtained from the insemination of flow sorted cryopreserved stallion spermatozoa. When comparing the results from the 4 treatments, it appears that damage to stallion spermatozoa induced by flow sorting is additional to that caused by freezing and thawing. This is evident when comparing the pregnancy rates of flow sorted frozen/thawed spermatozoa (13%) to that of nonsorted frozen spermatozoa (38%) or fresh flow sorted spermatozoa (38%). These differences would probably be greater if larger numbers of mares were used. It is imperative that a less harmful method of freezing stallion spermatozoa be developed if flow sorted frozen/thawed spermatozoa is to become practical.

The cause of the late term abortion in one mare was not known. A phenotypically normal fetus was recovered from the mare. Necropsy of the fetus reflected normal development to the time of pregnancy loss. Blood samples recovered from the mare and fetus were analysed and no abnormalities were detected.

In summary, we have demonstrated, for the first time, that pregnancies in mares can be obtained using only 5×10^6 flow sorted, frozen/thawed stallion spermatozoa when sperm are placed on and around the uterotubal junction by hysteroscopic insemination. Furthermore, hysteroscopic insemination can be used effectively to inseminate low numbers of frozen/thawed stallion spermatozoa as well as flow sorted stallion spermatozoa, and reasonable pregnancy rates can be achieved.

Manufacturers' addresses

[1]Animal Reproduction Systems, Chino, California, USA.
[2]Cytomation Inc., Fort Collins, Colorado, USA.
[3]Cook Veterinary Products, Brisbane, Queensland, Australia.
[4]Intervet Inc., Delaware, Ohio, USA.
[5]Bayer Corporation, Agriculture Division, Shawnee Mission, Kansas, USA.
[6]Pontax, Orangeburg, New York, USA.

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Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa

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Keywords: horse; low dose insemination; mares; sexed semen

Summary

The objectives of this study were 1) to compare pregnancy rates resulting from 2 methods of insemination using low sperm numbers and 2) to compare pregnancy rates resulting from hysteroscopic insemination of 5×10^6 nonsorted and 5×10^6 spermatozoa sorted for X- and Y-chromosome-bearing populations (flow sorted). Semen was collected with an artificial vagina from 2 stallions of known acceptable fertility. Oestrus was synchronised (June to July) in 40 mares, age 3–10 years, by administering 10 ml altrenogest orally for 10 consecutive days, followed by 250 μg cloprostenol i.m. on Day 11. All mares were given 3000 iu hCG i.v. at the time of insemination to induce ovulation. Mares were assigned randomly to 1 of 3 treatment groups: mares in *Treatment 1* (n = 10) were inseminated with 5×10^6 spermatozoa deposited deep into the uterine horn with the aid of ultrasonography. Mares in *Treatment 2* (n = 10) were inseminated with 5×10^6 spermatozoa deposited onto the uterotubal junction papilla via hysteroscopic insemination. Mares in *Treatment 3* (n = 20) were inseminated using the hysteroscopic technique with 5×10^6 flow sorted spermatozoa. Spermatozoa were stained with Hoechst 33342 and sorted into X- and Y-chromosome-bearing populations based on DNA content using an SX MoFlo sperm sorter. Pregnancy was determined ultrasonographically at 16 days postovulation. Hysteroscopic insemination resulted in more pregnancies (5/10 = 50%) than did the ultrasound-guided technique (0/10 = 0%; $P<0.05$) when nonsorted sperm were inseminated. Pregnancy rates were not significantly lower ($P>0.05$) when hysteroscopic insemination was used for sorted (5/20 = 25%) and nonsorted spermatozoa (5/10 = 50%). Therefore, hysteroscopic insemination of low numbers of flow sorted stallion spermatozoa resulted in reasonable pregnancy rates.

Introduction

High speed sperm sorting using flow cytometry has been used successfully to produce normal offspring in horses (Buchanan *et al.* 2000), cattle (Seidel *et al.* 1999), sheep (Cran *et al.* 1997), rabbits (Johnson *et al.* 1989) and man (Fugger 1999), as well as in several other species. Improvements to the sorting process and new methods of insemination are being developed to enhance the application of this new technology.

The most limiting factor in optimising the success of artificial insemination with flow sorted spermatozoa is the low number of sperm available after sorting. The minimal recommended dose for conventional artificial insemination in the mare is 500×10^6 progressively motile sperm (Pickett *et al.* 1989). Due to the current sort rate of around 1000 spermatozoa/s, it would take several days to obtain the recommended dose of spermatozoa for artificial insemination. This is not only impractical, but the viability of the spermatozoa would also be significantly reduced. Therefore, low dose insemination techniques must be developed to reduce the number of spermatozoa needed to maximise fertility (Buchanan *et al.* 2000; Morris *et al.* 2000).

The use of the videoendoscope as part of the clinical examination of the mare's reproductive tract (Brecher and Allen 1992) has enabled a relatively simple, rapid and atraumatic procedure to be developed for the deposition of low numbers of spermatozoa directly onto the papilla of the uterotubal junction (Morris *et al.* 2000). Hysteroscopic insemination of as few as 5×10^6 spermatozoa onto the papilla of the uterotubal junction resulted in a pregnancy rate of 75%, which is similar to that obtained with conventional intrauterine artificial insemination (Morris *et al.* 2000). This dose, 1/100th of that used for conventional uterine insemination, represents a sufficiently small number of spermatozoa that can be sorted into X- and Y-chromosome-bearing fractions in a reasonable time frame. At current sorting rates of 2.5×10^6 cells/instrument (MoFlo)^1/h, enough spermatozoa (either X- or Y-chromosome-bearing) for hysteroscopic insemination could easily be sorted within 4 h. A similar insemination method for low numbers of flow sorted ram spermatozoa has been used to produce successful pregnancies in ewes (Cran *et al.* 1997).

The 2 objectives of this study were 1) to compare pregnancy rates after insemination of 5×10^6 spermatozoa deep into the uterine horn with the aid of ultrasonography, with those obtained after deposition of the spermatozoa onto the papilla of the uterotubal junction using hysteroscopy and 2) to compare the pregnancy rates of mares inseminated by hysteroscopy with either 5×10^6 nonsorted or 5×10^6 flow sorted spermatozoa.

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EXHIBIT J

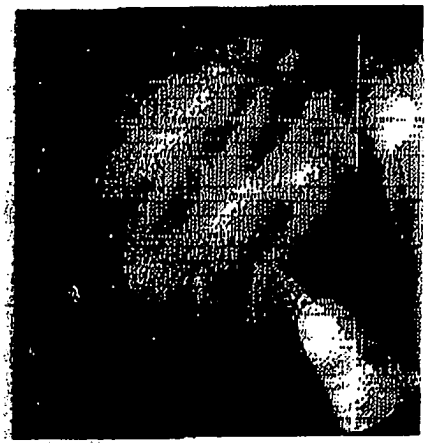

Fig 1: Inner and outer catheters both extruded from the videoendoscope. Inner catheter is in contact with uterotubal papilla.

Fig 2: The sperm suspension has been expelled onto the uterotubal papilla.

Materials and methods

Mare management

The oestrous cycles of 34 light horse type mares in good body condition, age 3–10 years, were synchronised by administering a synthetic progestagen, altrenogest (0.044 mg/kg bwt *per os*; Regumate)[2] daily for 10 consecutive days. Luteolysis was induced with the prostaglandin analogue, cloprostenol (250 μg Estrumate[3], i.m.), administered on Day 11. The mares' ovaries were examined ultrasonographically every second day until a follicle ≥30 mm diameter was detected. The mares with large follicles were then examined each morning until a follicle ≥35 x 35 mm was detected. These mares were assigned randomly to one of 3 treatment groups and inseminated the same afternoon.

Ovulation was induced in all mares by the administration of 3000 iu hCG (Chorulon)[4], administered i.v. at the time of insemination or up to a maximum of 8 h previously. Each mare was inseminated only once on the side ipsilateral to the ovary containing the dominant follicle. Following insemination, mares were examined daily by ultrasound until ovulation was detected to determine the day of ovulation. Pregnancy examinations were determined ultrasonographically on Days 12, 14, 16, 25 and 35 after ovulation (day of ovulation = 0). Pregnancy status was determined using Day 16 examination.

Three mares that became pregnant after insemination with flow sorted sperm were allowed to foal to determine the normalcy of offspring resulting from flow sorted spermatozoa, as well as to confirm the resulting sex of the foals.

Semen collection and processing

Two Arabian stallions (age 4 and 6 years) of acceptable fertility were used in this experiment. Semen was collected using an artificial vagina (CSU)[5] with an inline gel filter from each stallion on alternate days throughout the duration of the project. After collection, the semen was evaluated for gel-free volume, motility and sperm concentration. Samples were extended at a ratio of 10:1 (extender:semen, v/v) with prewarmed HBGM-3 (Parrish *et al.* 1988) and centrifuged immediately at room temperature for 15 min at 400 *g* to concentrate the spermatozoa. After centrifugation, the supernatant containing 90% of the seminal plasma was removed, leaving a soft sperm pellet for further processing for one of 3 sperm treatment groups.

Treatment 1: After centrifugation, the semen was diluted to provide 100 x 10^6 spermatozoa/ml in a commercial skim milk extender (EZ-Mixin CST)[5]. The sperm suspension was protected from light and maintained for 6 h at room temperature (20–25°C) to simulate the time needed to sort the spermatozoa for *Treatment 3*. The sperm suspension was then centrifuged through a 45:90% Percoll[6] discontinuous density gradient to reconcentrate the cells and to select a highly motile fraction of spermatozoa. The 90% Percoll was diluted at a ratio of 1:1 (v/v) with HEPES-buffered Tyrode's medium (Grandahl *et al.* 1996) to make a 45% solution. In a 15 ml centrifuge tube, 1 ml 45% Percoll was layered carefully on top of 1 ml 90% Percoll, then 1 ml of the sperm suspension (100 x 10^6 sperm/ml in E-Z Mixin CST) was layered on top of the Percoll layers, and the tube was centrifuged at 800 *g* for 12 min. After centrifugation, the supernatant was removed completely and the pellet resuspended in 600 μl HEPES-buffered Tyrode's medium. The sperm concentration was determined using a Densimeter (534B MOD-1)[7], and the required volume to deliver 5 x 10^6 spermatozoa (~100 μl) was calculated and prepared for insemination.

Mares (n = 10) were inseminated with 5 x 10^6 fresh, nonsorted spermatozoa using an ultrasound-guided technique (Buchanan *et al.* 2000). The inseminate was deposited at the cranial tip of the uterine horn ipsilateral to the preovulatory follicle using a disposable implant gun designed for use with 0.5 ml straws[7]. The location of the implant gun within the uterus was confirmed by transrectal ultrasonography prior to sperm deposition (Buchanan *et al.* 2000).

Treatment 2: Mares (n = 10) were inseminated with 5 x 10^6 fresh, nonsorted sperm using the hysteroscopic insemination technique (Morris *et al.* 2000). The inseminate was prepared by the same method used for *Treatment 1*. The predetermined dose was then aspirated into an equine GIFT catheter[8] using a 6 ml disposable syringe attached to the injection port on the distal end of the catheter. The loaded catheter was drawn into an outer polypropylene cannula, which was then passed down the working channel of a Pentax EPM 3000 videoendoscope[9]. The flexible endoscope (1.6 m long with an outer diameter of 12 mm) was

TABLE 1: Pregnancy rates for mares inseminated with nonsorted and flow sorted spermatozoa

Treatment	Mares inseminated	Mares pregnant 16 days (% pregnant)	Sperm inseminated ♂	Sperm inseminated ♀	Resulting sex ♂	Resulting sex ♀
Ultrasound, nonsorted	10	0 (0)[a]	--	--	--	--
Hysteroscopy, nonsorted	10	5 (50.0)[b]	--	--	--	--
Hysteroscopy, sex-sorted	20	5 (25.0)[a,b]	5	2	2[c]	2

[a,b]Values with different superscripts differ (P<0.05); [c]One pregnancy was lost prior to sex determination.

guided through the cervix and propelled forward through the uterine lumen of the mare (Bracher and Allen 1992). Under visual control the endoscope was directed along the uterine horn ipsilateral to the ovary containing the preovulatory follicle. When the tip of the endoscope came to within 3–5 cm of the papilla at the uterotubal junction, the outer cannula, followed by the inner GIFT catheter containing the sperm suspension, was extruded from the working channel of the endoscope until the tip of the GIFT catheter touched the papilla (Fig 1). The plunger of the syringe was then depressed, depositing the small volume (~100 µl) of the inseminate onto the surface of the papilla (Fig 2). The endoscope was withdrawn steadily from the uterus while simultaneously evacuating the filtered air that had previously been introduced to facilitate passage of the instrument through the uterine lumen.

Treatment 3: Mares (n = 20) were inseminated with fresh flow sorted spermatozoa using the hysteroscopic technique described for *Treatment 2*. The concentration of the spermatozoa after centrifugation was determined using the Densimeter, and a volume of HBGM-3 was added to provide a sperm concentration of 400 x 10^6 spermatozoa/ml. One ml of the sperm suspension was stained with 25 µl Hoechst 33342 (5 mg/ml DDH_2O) and incubated for 1 h at 34°C. The stained samples were then diluted to 100 x 10^6 sperm/ml for sorting by the addition of 3 ml HBGM-3 containing food colouring (2 µl/ml 1% FD&C No. 40). The samples were then filtered through a 40 µm filter into 6 ml polypropylene tubes and held at room temperature until further use (Johnson 1997). Argon lasers, emitting 150 mW at wavelengths of 351 and 364 nm, were used on each of 2 Cytomation MoFlo flow cytometer/cell sorters modified for sperm sorting at 50 psi. The sheath fluid was HBGM-3 without BSA. Spermatozoa were sorted at approximately 1000 live spermatozoa/s into 50 ml centrifuge tubes. For 14 mares, the flow sorted spermatozoa were collected into tubes containing 4 ml of a commercial skim milk extender (E-Z Mixin CST) as catch fluid. The remaining 6 mares were inseminated with spermatozoa collected into tubes containing 4 ml of skim milk and egg yolk extender (Squires *et al.* 1999). Tubes containing spermatozoa of corresponding sex were pooled from each flow cytometer, and sorted sperm were centrifuged for 20 min at 850 *g* at 22°C. The supernatant was removed to leave an ~200 µl sperm pellet. Pellets were resuspended in 100 µl HEPES-buffered Tyrode's medium containing 6% BSA. The final sperm concentration was calculated after counting the spermatozoa using a haemacytometer. The samples were then diluted to a final concentration of 50 x 10^6 spermatozoa/ml in HEPES-buffered Tyrode's medium containing 6% BSA. The predetermined volume (100 µl) was then loaded into an equine GIFT catheter and inseminated using the same hysteroscopic technique as described for *Treatment 2*.

Statistics

Statistical differences (P<0.05) in pregnancy rates were detected using Chi-square analysis of the results.

Results

No significant difference in pregnancy rates between the stallions was detected (Stallion A = 6/19, 31.5%; Stallion B = 4/21, 19%) and the data were therefore combined. Mares inseminated with the aid of the videoendoscope had significantly higher (P<0.05) pregnancy rates than those inseminated at the cranial tip of the uterine horn using the ultrasound-guided technique (Table 1).

None of the mares inseminated using the ultrasound-guided technique became pregnant compared with 5 out of 10 mares inseminated with nonsorted spermatozoa using the videoendoscope. Pregnancy rates were not significantly different for mares inseminated with fresh nonsorted sperm (50%) and flow sorted sperm (25%) after insemination using the videoendoscope.

One mare inseminated with Y-bearing sperm lost her pregnancy by 35 days after ovulation and therefore the sex of the fetus could not be determined. This was the only embryonic loss that occurred (1/5; 20%) resulting from flow sorted sperm. Pregnancies resulting from the insemination of nonsorted spermatozoa were terminated on Day 16, therefore embryonic loss rates cannot be compared between treatment groups.

One mare inseminated with X-chromosome-bearing sperm was subjected to euthanasia 18 days after ovulation due to a gastrointestinal problem. The conceptus was flushed prior to euthanasia of the mare and PCR analysis (Peippo *et al.* 1995) revealed it as a female, the expected sex. The remaining 3 mares foaled, and all correctly corresponded in sex to the spermatozoa inseminated. Therefore, of the 4 mares impregnated with flow sorted sperm, all foals (100%) were of the correct predetermined sex (Table 1).

Discussion

Based on the results of this experiment, hysteroscopic insemination directly onto the papilla of the uterotubal junction was the preferred method for insemination of mares not only with low numbers of spermatozoa (Morris *et al.* 2000) but also with low numbers of flow sorted stallion spermatozoa. Pregnancies were established in 5/20 inseminations (25%) using only 5 x 10^6 sorted, and in 5/10 insemination (50%) using the same number of nonsorted spermatozoa. On the other hand, no pregnancy was obtained after insemination with the ultrasound-guided technique (Buchanan *et al.* 2000) using similarly low numbers of nonsorted sperm. The results of the ultrasound-guided deep intrauterine insemination technique differ from those obtained originally by Buchanan *et al.* (2000), who achieved a 35% pregnancy rate (7/20) using 5 x 10^6 nonsorted spermatozoa. The reason for this difference is unclear, but it may be due to the additional sperm processing through the Percoll gradient and the lower volume of

the inseminate used in the present experiment, or it may be due to the use of different stallions and/or technicians. It is speculated that the low volume (~100 µl) of the inseminate used for the hysteroscopic insemination has a beneficial effect of maintaining the spermatozoa on the uterotubal junction. However, for deep insemination, a higher volume (~500 µl) may be required to facilitate passage of the spermatozoa to the site of fertilisation in the oviduct.

There appeared to be several advantages to using the hysteroscopic insemination technique. Firstly, the inseminators were able to deposit spermatozoa onto the uterotubal papilla more precisely, thereby minimising loss of the inseminate into the endometrial folds and deep crypts found in the uterus of the oestrous mare. In contrast, when the deep intrauterine technique (Buchanan *et al.* 2000) was used, the inseminators could not be sure of the precise location of sperm deposition. Even though the location of the tip of the pipette was observed ultrasonographically, this provided only a rough estimation of the exact location of the pipette at the time of insemination since the uterotubal junction could not be visualised by ultrasound. In addition, the location of the uterotubal junction was observed to be quite variable during hysteroscopy, which might decrease the precision of semen deposition using the ultrasound-guided technique.

Furthermore, while guiding the endoscope through the lumen of the mare's uterus, the inseminators could minimise damage to the uterine wall. However, in the ultrasound-guided technique (Buchanan *et al.* 2000), passage of the pipette through the lumen of the uterus relied solely upon manipulation of the pipette and the uterine horn *per rectum*, and irritation and damage to the endometrial wall may have occurred. This damage may create an inflamed uterine environment, which would be detrimental to both the sperm viability and subsequent embryonic development.

Nevertheless, potential problems may still arise during the hysteroscopic procedure. For example, the endoscope can easily become twisted during passage in the uterus of the mare, resulting in a disorientated video image, and the spermatozoa may be accidentally deposited in the uterine horn contralateral to impending ovulation. Obviously, accidental placement of the semen into the contralateral horn by deep uterine insemination is very unlikely. However, in this study, data from one mare was excluded after the tip of the videoendoscope was determined per rectal palpation to be in the contralateral uterine horn. From this experience, we believe that it is necessary to verify the location of the scope within the uterus, *per rectum*, prior to sperm deposition.

Furthermore, additional care must be taken to avoid postinsemination endometritis when utilising the videoendoscope. In our experiment, only one mare was found to have uterine inflammation following insemination. The low incidence of endometritis observed in this study could have been due to strict attention to vulval hygiene, cleaning of the endoscope between mares, use of a very small volume of semen and insemination of the mares prior to ovulation. It is also necessary to remove the air from the lumen of the uterus immediately after insemination to reduce the irritating effects that air may have on the endometrium (Caslick 1937).

There were no significant differences in the pregnancy rates of mares inseminated with nonsorted and flow sorted sperm in either the present study or the original ultrasound-guided insemination technique study (Buchanan *et al.* 2000). It must be realised, however, that differences in pregnancy rates may not have been evident due to the small number of mares used in these studies. Recent field trials utilising large numbers of cattle have shown similar pregnancy rates with both sorted and nonsorted sperm (Seidel *et al.* 1999). When the results were combined from all previous trials including the insemination of heifers with either flow sorted or nonsorted control spermatozoa, the pregnancy rates obtained after insemination of sorted sperm were within 90% of the nonsorted controls (Johnson and Welch 1999).

Buchanan *et al.* (2000) revealed a trend toward higher early embryonic loss rates in mares inseminated with flow sorted sperm than in the control group. In the present study, one of 5 mares (20%) inseminated with flow sorted spermatozoa lost the pregnancy at 24 days after ovulation. This mare developed an embryonic vesicle that had a normal appearance at Day 16; however, on Day 22, an abnormally small conceptus was detected which contained a fetus, but no heartbeat. By Day 24, no vesicle was present. When using fresh nonsorted semen, early embryonic loss rates have been reported to be 9% by Day 14 and as high as 16% between Days 20 and 50 (Squires 1998). Embryonic death rates in cattle have not increased after insemination with flow sorted spermatozoa (Seidel *et al.* 1999). When mares were inseminated with flow sorted spermatozoa using the ultrasound-guided technique, 3 of 8 mares (38%) lost their pregnancies between 16 and 60 days after ovulation (Buchanan *et al.* 2000). It is unlikely that this increase in embryonic loss was due to the sorting process, as it was not repeated in the present study. Rather, the loss may have been due to inflammatory changes associated with endometrial damage incurred during the deep intrauterine insemination procedure. Further studies involving higher numbers of mares inseminated with flow sorted sperm are needed to determine if embryonic loss will be greater for mares inseminated with flow sorted spermatozoa than pregnancy rates of those inseminated with nonsorted sperm.

Another factor that needs further investigation, in order to increase the efficacy of inseminating mares with flow sorted sperm, is determination of the appropriate number of sorted spermatozoa required for satisfactory fertility (Buchanan *et al.* 2000; Morris *et al.* 2000). The ideal insemination dose for flow sorted stallion spermatozoa will involve the lowest possible number of spermatozoa that can be used routinely to produce fertility rates within 90% of those rates resulting from conventional artificial insemination. Current pregnancy rates from insemination with flow sorted spermatozoa in the horse at 16 and 60 days after ovulation are, respectively, 25 and 20% when using 5×10^6 motile sperm (this study) and 40 and 25% when using 25×10^6 total sperm (Buchanan *et al.* 2000). Since these experiments utilised different insemination techniques, a comparison between the 2 studies is not justified. In a recent study using hysteroscopic insemination technique, Morris *et al.* (2000) reported that insemination of only 1×10^6 Percoll-treated nonsorted spermatozoa at the uterotubal junction resulted in satisfactory pregnancy rates (64%), which were equivalent to those obtained by conventional intrauterine insemination. It is possible that a similar insemination dose could be used successfully with flow sorted sperm, but further experiments are necessary to determine the minimal sperm number.

In this study, the inseminations were performed some 30–36 h prior to the anticipated time of ovulation. Further investigation to determine the optimum time of insemination using flow sorted spermatozoa is required. A major difference between the nonsorted hysteroscopic sperm treatment (2) and the flow sorted hysteroscopic sperm treatment (3) pertains to sperm processing

prior to insemination. The flow sorted spermatozoa were processed over several hours and were run through a flow cytometer. This should be compared with nonsorted control sperm, which was maintained in a skim milk-based extender for an equivalent time period, and then centrifuged for 12 min through a Percoll density gradient. It may be that insemination of the flow sorted sperm at a time closer to ovulation, to compensate for reduced sperm longevity, would improve pregnancy rates.

In summary, hysteroscopic insemination is a practical technique for insemination of low numbers of flow sorted stallion spermatozoa. Hysteroscopic insemination at the uterotubal junction is a relatively noninvasive and straightforward procedure which can be undertaken in the majority of mares and could, therefore, easily be incorporated into many modern breeding centres. The use of this technology, coupled with high-speed flow cytometric sorting (Johnson *et al.* 1989) in this trial, has produced 3 healthy, normal foals of the correct predetermined sex using only 5×10^6 motile spermatozoa. With continuing advances being made in these research areas, it is conceivable that this technology could be made available to commercial artificial insemination programmes in the near future.

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Manufacturers' addresses

[1]Cytomation Inc., Fort Collins, Colorado, USA.
[2]Hoechst Roussel Vet. Warren, New Jersey, USA.
[3]Bayer Corporatioin, Agricultural Division, Shawnee Mission, Kansas, USA.
[4]Intervet Inc., Millsboro, Delaware, USA.
[5]Animal Reproduction Systems, Chino, California, USA.
[6]Sigma Chemical Co., St Louis, Missouri, USA.
[7]Veterinary Concepts, Green Valley, Wisconsin, USA.
[8]Cook Veterinary Products, Brisbane, Australia.
[9]Pentax Ltd UK, Slough, Buckinghamshire. UK.

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SPERM CELL PROCESS SYSTEM

I. BACKGROUND

Effective preselection of sex has been accomplished in many species of livestock following the development of safe and reliable methods of separating sperm cells into enriched X chromosome bearing and Y chromosome bearing populations. Separation of X chromosome bearing sperm cells from Y chromosome bearing sperm cells can be accomplished as disclosed herein and as disclosed by various international patent applications, for example: PCT/US99/17165; PCT/US98/27909; PCT/US01/45237; PCT/01/18879; PCT/US01/15150; and PCT/US01/02304 and United States patent applications 09/582,809; and 09/015,454, each hereby incorporated by reference. These examples of separating X chromosome bearing sperm cells from Y chromosome bearing sperm cells are not meant to limit the instant sperm cell process system invention to sperm processing technology that flow cytometry sorting devices or flow-sorting methods but rather are meant to be illustrative of various processes by which sperm cells may be separated from one another and to be illustrative of the manner in which sperm cells are collected, handled, separated, transported, used, or stored as a context in which the instant invention can be understood.

Even though the various devices and methods of separating sperm cells have improved significant problems remain with respect to maintaining sperm viability during collection, handling, transportation, separation, use, or storage processes.

II. SUMMARY OF THE INVENTION

Accordingly, the broad object of the invention can be to provide devices or methods for the collection, handling, shipment, storage, or separation of semen or sperm cells to maintain sperm viability.

Another broad object of the invention can be to provide devices or methods for collecting, handling, shipment, storing, or separating semen or sperm cells obtained from

EXHIBIT K

[redacted]

various species of mammals, including, but not limited to equids, bovids, felids, ovids, canids, buffalo, oxen, elk, or porcine; or obtained from prize, endangered, or rare individuals of a mammal species; or obtained from zoological specimens to maintain or enhance sperm viability.

Another significant object of the invention can be to provide devices or methods for handling and transporting sperm cells obtained from equine mammals.

Another significant object of the invention can be to provide devices or methods of separating sperm cells that can maintain greater viability of mamalian sperm cells throughout a flow-sorting process.

Another significant object of the invention can be to provide devices or methods of maintaining sperm cells at greater viability for purposes of artificial insemination of various species of mammals, such as those described above, or even artificial insemination with a low or reduced number of sperm cells compared to the usual number or typical number of sperm cells used in such artificial insemination procedures whether or not such sperm cells are separated into enriched X chromosome bearing or Y chromosome bearing sperm cells.

Another significant object of the invention can be to provide devices or methods for the shipment of stallion sperm prior to separation of flow-sorting of sperm cells.

III. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a graph showing that as stain concentration increases the total and progressive motility of sperm cells decreases, % dead sperm cells increases, and the ability of flow cytometry techniques to resolve X-chromosome bearing sperm cells from Y-chromosome sperm cells decreases.

Figure 2 provides a graph showing that sperm cells extended in KMT remain more motile with respect to both fresh sperm samples and sperm samples stored for a duration of time at room temperature, such as 18 hours at room temperature

Figure 3 provides a graph showing that staining sperm cells at higher pH can decrease the % dead sperm cells in stained sperm cells samples as evaluated by flow cytometry analysis.

Figure 4 provides a graph showing that decreasing the stain incubation period from a conventional period of 60 minutes to about a 30 minute incubation period can increase motility, decrease % dead sperm cells in stained sperm cell samples, and increase resolution of X-chromosome bearing sperm cells from Y-chromosome bearing sperm cells during flow sorting of stained sperm cells.

Figure 5 provides a graph showing that the addition of a stimulant, such as caffeine, can increase motility in sperm cells.

Figure 6 provides a graph showing that total motility and progressive motility of sperm cells can be increased using modified KMT prepared using NaH_2PO_4.

Figure 7 provides a graph showing that total motility and progressive motility of sperm cells can be increased using modified KMT prepared using NaH_2PO_4 whether or not the sperm cells are exposed to stimulant, such as caffeine.

Figure 8 provides a graph showing that temperature can be adjusted for storing, handling, transferring, or transportation of sperm cells obtained from a male of a species of mammal to increase total and progressive motility.

Figure 9 provides a graph showing that the temperature at which sperm cells are transferred, stored, or handled prior to a staining protocol can be adjusted to increase total or progressive motility of sperm cells, or stimulated sperm cells, or sperm cells stimulated with caffeine.

Figure 10 provides a graph showing that temperature at which sperm cells are transferred, stored, or handled prior to a staining protocol can be adjusted to increase total or progressive motility of sperm cells, or stimulated sperm cells, or sperm cells stimulated with caffeine subsequent to a staining protocol.

Figure 11 provides a graph showing that % dead sperm cells in stained sperm cell samples can be reduced by storing or transporting sperm cells at 15°C.

Figure 12 provides a graph showing that sperm cells can remain more viable when sperm cell concentration during staining is at about 100 M/mL versus 200M/mL without loss of flow cytometry resolution.

Figure 13 provides a graph showing that as stain concentration increases fewer sperm cells survive and resolution decreases

Figure 14 provides a graph showing that stain time can be substantially decreased without loss of resolution between X-chromosome bearing populations and Y-chromosome bearing populations of sperm cells evaluated by flow cytometery.

IV. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A semen or sperm cell process system to maintain or enhance the biological, chemical, physical, physiological, or functional attributes of sperm cells within the context of various collecting, handling, storage, transportation, separation, or insemination procedures.

EXAMPLE 1.

Semen was collected from three stallions of acceptable fertility, extended to about ~25 x 10^6 sperm/mL in a Tyrode's-based skim milk-glucose extender, and stored for 18 h

at either about 5°C or about 15°C. Following storage, spermatozoa were centrifuged to remove seminal plasma and concentrate sperm, stained with Hoechst 33342 (Hoechst), and sorted into enriched X-chromosome bearing and Y-chromosome-bearing populations based on DNA content using an SX MoFlo® sperm sorter.

A final dose of about 20 x 10^6 flow-sorted sperm in a volume of 300 µL was used for all inseminations. Estrus was synchronized in 35 mares ages 2 to 12. Human chorionic gonadotropin (hCG; 3000 IU, iv; Chorulon®, Intervet, Millsboro, DE, USA) was administered when a dominant follicle ≥35 mm in diameter was present and mares were inseminated at approximately 30 h post-hCG. At the time of insemination, mares were assigned to 1 of 3 treatment groups: 1) sperm that had been stored at 15°C and inseminated using the videoendoscopic technique; 2) sperm stored at 5°C and also inseminated using the videoendoscopic method; and 3) sperm stored at 5°C and inseminated using the rectally guided technique.

Mares were sedated immediately prior to insemination using butorphanol (4 mg, iv; Torbugesic®, Ft. Dodge Co., Fort Dodge, IA, USA) and detomidine (6 mg, iv; Dormosedan®, Pfizer, Lees Summit, MO, USA). Mares were evaluated daily for ovulation, and only those mares ovulating within 48 h after insemination were included in the results. Pregnancy was determined ultrasonographically at 12 to 14 days post-ovulation. Mares were administered prostaglandin $F_2\alpha$ at day 16 post-ovulation for use in 2 subsequent cycles.

Pregnancy rates between mares inseminated hysteroscopically with sorted sperm stored at 15°C was about 72%, or at 5°C was about 55% as shown by Table 1. There was a tendency (P=0.12) for fewer mares to become pregnant following rectally guided insemination (38%) compared to hysteroscopic insemination (55%) when stored, sorted sperm were inseminated.

Table 1. Pregnancy rates from hysteroscopic or rectally guided insemination of flow-sorted sperm stored at 5°C or 15°C.

Storage temp. (°C)	Insemination method	Mares inseminated	Mares pregnant (14 d)	Pregnancy rate
15	Hysteroscopic	25	18	72%[a]
5	Hysteroscopic	22	12	55%[a,b]
5	Rectally guided	24	9	38%[b]

[a,b] Values in the same column with different superscripts are significantly different ($P<0.05$).

A greater number of mares became pregnant following insemination with sorted sperm stored prior to sorting at 15°C as compared to 5°C. This effect was consistent across stallions as was fertility after storage at 15°C for 18 h prior to sorting, as compared to 5°C.

The expected pregnancy rate following insemination of 1×10^9 stallion sperm shipped by standard methods (5°C, 12 to 72 h) is 65%. The pregnancy rate obtained in the present study (72%) is impressive, and shows dramatic improvement over that obtained (35%) with 18 h stored, flow-sorted sperm using conventional technology. This increase in fertility may reflect sperm processing prior to flow cytometry. Pregnancy rates in the present study would have been even higher except that for two days during this application of the invention pregnancy rates for all mares inseminated were extremely low.

Hysteroscopic insemination resulted in higher pregnancy rates than deep-uterine insemination. This is in contrast to Rigby et al. who reported similar pregnancy rates between videoendoscopic and rectally guided insemination when shipped sperm were used. However, those results did show a 12-percentage point advantage for hysteroscopic insemination. In contrast to that study, the present trial utilized flow-sorted sperm, which are known to be in a pre-capacitated state. In summary, excellent pregnancy rates were obtained with hysteroscopic insemination of 18 h stored, flow-sorted spermatozoa.

Pregnancy rates were higher for all three stallions when sperm was maintained at about 15°C as compared to 5°C. Hysteroscopic insemination with sperm stored at 15°C resulted in higher pregnancy rates than rectally guided insemination of sperm stored at 5°C.

As such, the sperm cell process system invention can involve obtaining sperm cells from a male of a species of mammal, maintaining the sperm cells obtained from the male species of mammal prior to artificial insemination of a female of the species of mammal at a temperature(s) selected within the range of between 5°C and 25°C that generates a higher pregnancy rate of such females of such species of mammal. With respect to equine species, and particularly with respect to the species of equids disclosed, the temperature at which sperm cells obtained from equine males are maintained in accordance with the invention to increase pregnancy rate can be between about 10°C to about 20°C, and can specifically be about 15°C. See Examples below for application involving equids. See also, Example 8 below showing an application of the invention in which sperm cells obtained from a male of a species of elk are maintained at about 20°C prior to insemination of cow elk.

The sperm cell process system invention can further include transportation of the sperm cells obtained from the male of a species of mammal maintained at temperature(s) in accordance with the invention. Such transportation may have a limited duration of less than an hour or may have a more extended duration between about 1 hour and about 72 hours, or as described may be a duration of about 18 hours.

The invention can further include the step of staining the sperm cells obtained from a male of a species of mammal as above-described which have been maintained at a temperature that generates the highest pregnancy rate of such females of such species of mammal.

The invention can further include hysteroscopic or rectally guided artificial insemination of the female of the species of mammal. Specifically, as described by the

incorporation by reference included herein, hysteroscopic insemination of equine mammals with sperm cells sorted for sex preselection that may be handled in accordance with the instant invention and may further include a low number of sperm cells compared to the number of sperm cells typically used to inseminate a female of a particular species of mammal, including but not limited to equine mammals.

EXAMPLE 2.

Semen from eight stallions was extended to about 25 x 10^6 sperm/ml in each of four shipping extenders as set out in Table 2. During simulated shipping for 18 h, samples were held at ambient temperature (20-24°C), except those extended in INRA96, which were stored at 15°C. Following storage, samples were centrifuged at 600 x g for 10 min and pellets extended to 400 x 10^6 sperm/ml. After incubation at 19-24°C for 1 h and dilution to 200 x 10^6 sperm/ml, sperm were stained at 34°C with 224 µM Hoechst 33342 for 1 h, and then diluted to 100 x 10^6 sperm/ml in KMT. To simulate sorting conditions, sperm were diluted to 700,000 sperm/ml in HBGM-3 without BSA and held at ambient temperature for 1.5 h prior to centrifugation at 850 x g for 20 min. Motility was evaluated at four chemical environments as shown by Table 2.

Table 2. Percentage of motile sperm in samples stored in four different shipping media

Media	Post-ship	Pre-Stain	Post-Stain	Post- Dil
EZ Mixin CST; Anim Reprod Systems, Chino, CA	59	$48^{a,b}$	31^{b}	47^{a}
Next Generation; Exodus Breeders, York, PA	54	40^{b}	27^{b}	36^{b}
KMT; J Anim Sci 1991;69:3308-3313	64	58^{a}	46^{a}	50^{a}
INRA96; IMV Technologies, France	63	53^{a}	43^{a}	54^{a}

[a,b] Values in the same column without common superscripts differ (P<0.05), Tukey's Test.

As can be understood from the data set out by Table 2, KMT and INRA96 maintained higher motility throughout certain sperm cell process procedures.

The sperm cell process system invention can further include the step of extending sperm cells obtained from a male of a species of mammal in KMT, and specifically with respect to sperm cells obtained from the male of an equine species of mammal KMT can significantly increase sperm cell motility.

The sperm cell process system invention can further include the step of extending sperm cells obtained from a male of a species of mammal in INRA96, and specifically with respect to sperm cells obtained from the male of an equine species of mammal INRA96 can significantly increase sperm cell motility.

EXAMPLE 3.

Ejaculates from 8 stallions were extended to 25 x 10^6 sperm/ml in KMT, and 40-ml aliquots were placed at 5, 10, 15, 20, and 25°C for 18 h. Samples were then processed similarly to methods of Example 2. Motility was evaluated both with and without 2 mM caffeine as a stimulant. Flow-cytometric evaluation of % dead was done using propidium iodide staining.

Table 3. Percentage of motile sperm after 18 h at varying temperatures (°C) (non-stim./stim.).

Temperature	Post-Shipping	Pre-Stain	Post-Stain	Post-High Dilution	% Dead
5	$63^{ab}/63^{ab}$	$56^{a}/56$	$49^{a}/49^{a}$	$45^{a}/50^{ab}$	28^{ab}
10	$62^{ab}/63^{a}$	$58^{a}/56$	$48^{ab}/52^{a}$	$45^{a}/50^{ab}$	26^{ab}
15	$65^{a}/64^{a}$	$56^{a}/54$	$45^{ab}/51^{a}$	$45^{a}/51^{a}$	23^{a}
20	$59^{bc}/62^{ab}$	$54^{ab}/56$	$42^{b}/48^{ab}$	$41^{a}/47^{ab}$	23^{a}
25	$56^{c}/59^{b}$	$49^{b}/53$	$35^{c}/43^{b}$	$35^{b}/44^{b}$	31^{b}

[a,b,c] Values in the same column without common superscripts differ ($P<0.05$).

As shown by Table 3, using KMT can reduce motility difference with respect to storage or transportation temperatures of 5, 10, or 15°C storage temperatures.

As such, the sperm cell process system invention can further include the step of diluting or maintaining such sperm cells obtained from the male of the species of mammal in such concentration(s) of KMT prior to staining such sperm cells with Hoechst stain and prior to flow sorting of such sperm cells

EXAMPLE 4.

Sperm cells in ejaculates from three stallions were initially evaluated for volume, concentration, and motility. The remaining portion of the ejaculates were extended with either KMT or EZ mixin with either 0% additional seminal plasma or 10% seminal plasma by concentration to the following sperm cell/stain concentrations: 50 $x10^6$ sperm/mL, 2.6µl Hoechst; 50 $x10^6$ sperm/mL, 3.9µl Hoechst; 150 $x10^6$ sperm/mL, 7.8µl Hoechst; or 450 $x10^6$ sperm/mL, 23.4 µl Hoechst and processed either immediately or after 18 hours storage at room temperature. Stained sperm cell samples were then evaluated for resolution and % dead by flow cytometry analysis, and motility was evaluated by further diluting 20 µl of each stained sperm cell sample with 140 µl EZ Mixin or KMT.

Now referring primarily to Figure 1, it can be understood that as stain concentration increases the total and progressive motility of sperm cells, and in particular equine sperm cells, decreases, % dead sperm cells increases, and the ability of flow cytometry techniques to resolve X-chromosome bearing sperm cells from Y-chromosome sperm cells decreases.

The sperm cell process system invention can comprise a range of stain concentration(s) that provides enhanced total or progressive motility of stained sperm cells, resolution of X-chromosome bearing sperm cell from Y-chromosome bearing sperm cells during flow-sorting; or decrease in the % dead sperm cells, compared to the range of stain concentration used in conventional technology with respect to sperm cells obtained from a particular species, or other ranges of stain concentrations as disclosed. Specifically for equine applications of the invention, the range of stain concentration that can provide enhanced total or progressive, enhanced resolution of X-chromosome bearing sperm cell from Y-chromosome bearing sperm cells during flow-sorting; or a reduction of the % dead sperm cells in stained sperm cell samples can be between about 50 $x10^6$ sperm/mL, 2.6µl Hoechst; 50 $x10^6$ sperm/mL, 3.9µl Hoechst. While results are

less favorable the range of stain concentration can be between 150 x10^6 sperm/mL, 7.8µl Hoechst to about 450 x10^6 sperm/mL, 23.4 µl Hoechst.

Now referring primarily to Figure 2, it can be understood that sperm cells extended in KMT remain more motile with respect to both fresh sperm samples and sperm samples stored for a duration of time at room temperature, such as 18 hours at room temperature.

The invention can further include use of KMT as an extender to increase total or progressive motility of fresh sperm cells, of sperm cells stored for a duration of time, for example up to 18 hours or longer, or of sperm cells that are transferred or transported from a first location, such as the location at which the sperm cells are obtained from a male mammal, to a second or a plurality of locations where further processing of sperm cells obtained from the male mammal occurs, such as sperm cell counting, separation of X-chromosome bearing sperm cells from Y-chromosome bearing sperm cells, or preparation of sperm cell containing products including but not limited to the manufacture of straws of sperm for artificial insemination (whether sorted or not), or a second or plurality of locations where insemination of a female species of the mammal occurs, oocytes are fertilized in-vitro, or the like.

EXAMPLE 5.

Sperm cells in ejaculates from three stallions were initially evaluated for volume, concentration, and motility. The remaining portion of each ejaculate was extended in KMT to 25 x10^6 sperm/mL and stored at RT for 18 hr. The stored ejaculates were pelleted by centrifugation at 600g for 10 min. Pelleted sperm cells were resuspended in KMT with Hoechst to generate sperm samples of 400 x10^6 sperm/mL, 12.4 µl Hoechst, adjusted to either 7.1 pH or 7.9 pH, and then incubated at 34°C for either 30min or 60min. Stained sperm cell samples were extended with either 1mL KMT with 1.5 µl/mL 5% red food dye; 1mL KMT with 2.0 µl/mL 5% red food dye; 1mL KMT with 2.5 µl/mL

5% red food dye; or 1mL KMT with 3.0 µl/mL 2% red food dye. Sperm cell samples were then evaluated for % dead and resolution by flow cytometry analysis, and motility was evaluated by further diluting treated samples in either 140 µl KMT: 20 µl sperm cells; 140 µl KMT 2mM caffeine: 20 µl sperm cells; 140 µl KMT, 2.5 mM NaPyruvate: 20 µl sperm cells.

Table 4. Effect of Stallion

	Gunsmoke	Rowdy	Sylekt
% Dead	15.5	16	17.13
Resolution	5.81	4.5	5.88

	Gunsmoke	Rowdy	Sylekt
Motility 0h	55	68.44	59.69
Progressive 0h	43.13	68.44	56.25
Motility 3h-None	63.13	65.94	67.19
Motility 3h-Caffeine	66.25	67.5	69.38
Motility 3h-Pyruvate	62.19	67.19	65.94
Progessive 3h-None	55.94	65.94	63.75
Progressive 3h-Caffeine	63.13	67.5	69.06
Progressive 3h-Pyruvate	56.56	67.19	63.13

Table 5. Effect of Stain pH

	7.1	7.9
% Dead	17.25	15.17
Resolution	5.58	5.21

	7.1	7.9
Motility 0h	62.08	60
Progressive 0h	55.83	56.04
Motility 3h-None	66.25	64.58
Motility 3h-Caffeine	68.54	66.88
Motility 3h-Pyruvate	65.83	64.38
Progessive 3h-None	62.29	61.46
Progressive 3h-Caffeine	67.5	65.63
Progressive 3h-Pyruvate	63.13	61.46

Table 6. Effect of Food Coloring

	1.5	2	2.5	3
% Dead	16	16.33	16.17	16.33
Resolution	5.33	5.25	5.5	5.5

	1.5	2	2.5	3

Motility 0h	60.42	62.08	62.5	59.17
Progressive 0h	55.83	56.67	57.08	54.17
Motility 3h-None	64.58	65.42	66.25	65.42
Motility 3h-Caffeine	66.67	68.33	68.75	67.08
Motility 3h-Pyruvate	64.58	65	65.83	65
Progessive 3h-None	61.67	61.67	62.92	61.25
Progressive 3h-Caffeine	65.42	67.5	67.92	65.42
Progressive 3h-Pyruvate	62.08	60.83	64.58	61.67

Table 7. Effect of Stain Time (12 Samples only)

	30 min	60 min
% Dead	14.83	16.17
Resolution	4.92	5.5

	30 min	60 min
Motility 0h	66.25	62.5
Progressive 0h	62.08	57.08
Motility 3h-None	65.83	66.25
Motility 3h-Caffeine	66.67	68.75
Motility 3h-Pyruvate	64.58	65.83
Progessive 3h-None	62.08	62.92
Progressive 3h-Caffeine	65	67.92
Progressive 3h-Pyruvate	60.42	64.58

Table 8. pH and Stain Time

	7.1		7.9	
	30 min	60 min	30 min	60 min
% Dead	15.67	17	14	15.33
Resolution	4.83	5.83	5	5.17

	7.1		7.9	
	30 min	60 min	30 min	60 min
Motility 0h	65.83	62.5	66.67	62.5
Progressive 0h	61.67	55.83	62.5	58.33
Motility 3h-None	65.83	68.33	65.83	64.17
Motility 3h-Caffeine	66.67	70	66.67	67.5
Motility 3h-Pyruvate	64.17	67.5	65	64.17
Progessive 3h-None	63.33	64.17	60.83	61.67
Progressive 3h-Caffeine	65	70	65	65.83
Progressive 3h-Pyruvate	60.83	66.67	60	62.5

Table 9. Motility Stimulants

	None	Caffeine	Pyruvate
Motility 3h	65.42	67.71	65.1
Progressive 3h	61.88	66.56	62.29

Now referring primarily to Figure 3, it can be understood that staining sperm cells at higher pH can decrease the percent dead sperm cells as evaluated by flow cytometry analysis.

Now referring primarily to Figure 4, it can be understood that decreasing the incubation period to stain sperm cells from the conventional period of 60 minutes to a 30 minute period can increase motility, decrease percent dead sperm cells, and increase resolution of X-chromosome bearing sperm cells from Y-chromosome bearing sperm cells during flow cytometry.

Now referring primarily to Figure 5 it can be understood that the addition of a stimulant such as caffeine at a concentration of about 2mM can stimulate motility in sperm cells and can be particularly effective in stimulating stressed equine sperm cells.

As such, the invention can further include the step of adjusting the pH of the solution in which sperm cells obtained from the male of a species of mammal are stained with a flourochrome, such as Hoechst. The pH of the stain solution can be raised to a pH between about 7.2 pH to about 8.0 pH to select the pH desired for a particular sperm cell sample or the one that generates a reduced or least % dead in a particular type of stained sperm cell sample. Specifically, with respect to sperm cells obtained from equine males the pH of the stain solution can be raised to between about 7.5 pH to about 8.0 pH and specifically can be 7.9 pH to reduce the % dead equine sperm cells in stained sperm cell samples.

The invention can further include the step of reducing the period of time in which sperm cells are incubated in the stain solution to reduce the % dead in stained sperm samples or to increase motility, or increase resolution of X-chromomsome bearing stained sperm from Y-chromosome bearing stained sperm when flow sorted or otherwise separated based on DNA content. Depending on the application in which the invention is

employed, the period of time in which sperm cells are exposed to, incubated in, or are otherwise suspended in stain solution can be substantially reduced. The reduction in time can be 10%, 20%, 30%, 40%, 50%, or more from the amount of time typically used; or can be a reduction in time that reduces the number of dead sperm cells resulting from staining with a fluorochrome without a significant reduction in resolution during flow sorting; or can be a reduction in time that results in increased resolution during flow sorting without a significant increase in the % dead sperm cells in the stained sample. The amount of time that an equine sperm cell sample, for example, incubates in a staining solution of Hoechst (as described above) can be between about 25minutes to about 50 minutes to obtain greater flow sorting resolution or reduced % dead sperm cells, and can specifically be 30 minutes. The actual reduction in time can be determined to provide a desired balance between motility, % dead sperm cells, and flow sort resolution within a population of sperm cells proximate to the time of stained.

The invention can further include the step of adding a stimulant to the sperm cell sample. The stimulant can be caffeine, or a stimulant similar to caffeine, or a stimulant that increases sperm cell motility or other sperm cell function or characteristic, whether mechanical or physiological. The stimulant can be added prior to or after the sperm cell is exposed to a process step such as storage, transportation, dilution, flow sorting, insemination, or the like. Specifically, a concentration of between about 1mM and about 5mM caffeine can be used and specifically with respect to equine sperm cells a 2mM concentration of caffeine can be used.

EXAMPLE 6.

Ejaculates from ten stallions were initially evaluated for volume and sperm cell concentration and motility. The remaining portion of each ejaculate was extended in either KMT prepared using $Na_2H_2PO_4$ KMT or KMT prepared using NaH_2PO_4 (KMT-mod.) to 25 $x10^6$ sperm/mL and stored at 5°C, 10°C, 15°C, 20°C, 25°C for 18 hr. A 100 µl aliquot of treated sperm cells were then diluted with 100 µl KMT or 100 µl KMT, 4mM caffeine and motility of the sperm cells was evaluated. The remaining portion of

the treated sperm cells in each sample were centrifuged at 600g for 10 min. the supernatant aspirated to about 0.75 mL and the pelleted sperm cells resuspended in that volume. Post centrifugation motility of treated sperm cells was evaluated after dilution of 20 µl of each sample with either 520 µl KMT or 520 µl KMT, 2mM caffeine.

Aliquots of 200 x10^6 sperm/mL,12.4 µl Hoechst of each treatment group adjusted to 7.1 pH were incubated at 34°C for 60min. Stained sperm cell samples were extended with either 1mL KMT with 1.5 µl/mL 5% red food dye. High dilution samples were then prepared by addition of 3mL KMT or 3mL KMT-mod. and 22mL 5mMHBGM-3 into 175 µl stained sperm (at 100 x10^6 sperm/mL).

Treated sperm cell samples were then evaluated for % dead and resolution by flow cytometry analysis, and motility was evaluated by further diluting treating samples in either 140 µl KMT: 20 µl sperm cells; or 140 µl KMT 2mM caffeine: 20 µl sperm cells.

Table 10. Effect of Stallion

	Pship	Pcent	Pstain	Hdil	%dead	resolution
A	63.3	52.5	44.5	47.5	22.8	5.7
B	54.5	50.8	32.3	31.8	41.1	8.2
C	68.8	62.8	55.6	44.1	21.4	6.2
D	64.7	58.4	46.6	48.4	26.1	7.3
E	65.3	57.8	48.8	45.5	21.6	5.2
G	58.8	58	36.8	37	30.7	7
H	58	51.5	48.5	46.5	25.2	8
J	58	50	41.8	41.8	17.9	6.8

Table 11. Effect of Extender

	Pship	Pcent	Pstain	Hdil	%Dead	Resolution
KMT	60.7	52.8	42.8	40.9	26.6	6.8
KMT Mod	61.6	57.1	45.2	44.4	25.5	6.8

Table 12. Effect of Shipping Temperature

	Pship	Pcent	Pstain	Hdil	%Dead	Resolution
5	62.5	56.1	48.8	45	27.9	6.9
10	62	58	47.5	44.7	26.4	6.8
15	64.7	56.3	44.5	44.8	23.1	6.7
20	59.2	53.6	42.2	41.4	23.3	6.6
25	55.8	49.4	34.6	35.4	31.4	7.2

Table 13. Post-Ship Motility

	Total	Prog.	Stim.,	T Stim.,Prog
5	62.5	59.2	62.5	61.4
10	62	58.9	63.3	61.9
15	64.7	62.7	64.2	62.7
20	59.2	59.1	62.3	61.1
25	55.8	55	59	57.9

Table 14. Post-Centrifugation Motility

	Total	Prog.	Stim.	T Stim.Prog
5	56.1	55.6	56.3	55.2
10	58	56.3	56.4	54.4
15	56.3	56.1	53.6	52.2
20	53.6	52.8	56.4	55.6
25	49.4	48.5	52.7	51.9

Table 15. Post-Staining Motility

	Total	Prog.	Stim., T Stim.,	Prog
5	48.8	48.6	49.1	49.1
10	47.5	47.2	52	51.9
15	44.5	44.5	51.4	51.3
20	42.2,	42.2	48.1	47.5
25	34.6	34	42.7	42.7

Table 16. Post-High Dilution Motility

	Total	Prog.	Stim.,	T Stim.,Prog
5	45	45	49.5	49.5
10	44.7	44.1	49.5	48.9
15	44.8	44.8	51.1	51.1
20	41.4	40.5	46.9	46.9
25	35.4	36.9	44.4	43.3

Table 17. Percent Dead and Resolution

	% Dead	Resolution
5	27.9	6.9
10	26.4	6.8
15	23.1	6.7
20	23.3	6.6
25	31.4	7.2

Table 18. KMT vs mod, Post-ship motility

	KMT	mod
5	62.8	62.2
10	61.9	62.2
15	63.4	65.9
20	57.8	60.6
25	56.3	55.4

Table 19. KMT vs mod, High Dilution motility

	KMT	mod
5	42.8	47.2
10	44.4	45
15	43.1	46.6
20	40	42.8
25	31.7	39.2

Table 20. KMT vs KMT-mod, Percent dead

	KMT	mod
5	27.5	28.4
10	26.4	26.5
15	23.3	23
20	23	23.6
25	36.8	26

Now referring primarily to Figure 6, total motility and progressive motility of sperm cells can be increased using modified KMT prepared using NaH_2PO_4.

Now referring primarily to Figure 7 it can be understood that total motility and progressive motility of sperm cells after process steps such as staining for flow sorting and steps in which sperm cells are diluted can be increased using modified KMT prepared using NaH_2PO_4 whether or not the sperm cells are exposed to stimulant such as caffeine.

Now referring primarily to Figure 8 it can be understood that temperature can be adjusted for storing, handling, transferring, or transportation of sperm cells obtained from a male of a species of mammal to increase total and progressive motility. With respect to some sperm cells from certain species of mammals, storing, handling, transferring, or transportation at about 15°C can maintain highest levels of total or progressive motility of sperm cells or stimulated sperm cells.

Now referring to Figure 9, it can be understood that the temperature at which

sperm cells are transferred, stored, or handled prior to a staining protocol, such as described above, can be adjusted to increase total or progressive motility of sperm cells, or stimulated sperm cells, or sperm cells stimulated with caffeine. With respect to certain embodiments of the invention, including those in which equine sperm cells are processed, storage, transfer, or transport temperatures of between about 5°C to about 20°C can increase total and progressive motility of sperm cells. Moreover, with respect to stimulated sperm cells processed in accordance with the invention, including those embodiments of the invention in which equine sperm cells are stimulated with caffeine, handling, storage, or transfer temperatures between 5°C to about 20°C can also increase total and progressive motility. Specifically, embodiments of the invention used to process stimulated equine sperm cells comprise temperatures between about 10°C to about 15°C for handling, storing, or transferring of stimulated equine sperm cells.

Now referring to Figure 10, it can be understood that the temperature at which sperm cells are transferred, stored, or handled prior to a staining protocol, such as described above, can be adjusted to increase total or progressive motility of sperm cells, or stimulated sperm cells, or sperm cells stimulated with caffeine subsequent to a staining protocol. With respect to certain embodiments of the invention, including those in which equine sperm cells are processed, storage, transfer, or transport temperatures of between about 5°C to about 20°C can increase total and progressive motility of sperm cells subsequent to staining protocols. Moreover, with respect to stimulated sperm cells processed in accordance with the invention, including those embodiments of the invention in which include equine sperm cells stimulated with caffeine, handling, storage, or transfer temperatures between 5°C to about 20°C can also increase total and progressive motility. Specifically, embodiments of the invention used to process stimulated equine sperm cells comprise temperatures between about 10°C to about 15°C for handling, storing, or transferring of stimulated equine sperm cells.

Now referring to Figure 11, it can be understood that % dead of sperm cells after staining as described above can be reduced by storing or transporting sperm cells at 15°C.

EXAMPLE 7.

Ejaculates from twelve stallions were initially evaluated for volume and sperm cell concentration and motility. The remaining portion of each ejaculate was extended in KMT to 25 x10^6 sperm/mL and stored at 15°C for 18 hr. Post storage motility was evaluated using100 µl aliquots of treated sperm cells were then diluted with 100 µl KMT, 4mM caffeine. The remaining portion of the treated sperm cells in each sample were centrifuged at 600g for 10 min. the supernatant aspirated to about 1.50 mL and the pelleted sperm cells resuspended in that volume. Treated sperm cells were extended to 400 x10^6 sperm/mL and aliquots transferred to a staining tube for treatment as follows:

1. 200 x10^6 sperm/mL, 8.68 µl Hoechst 7.1 pH, incubated at 34°C for 60min
2. 200 x10^6 sperm/mL,10.54 µl Hoechst 7.1 pH, incubated at 34°C for 60min
3. 200 x10^6 sperm/mL,12.44 µl Hoechst 7.1 pH, incubated at 34°C for 60min
4. 200 x10^6 sperm/mL, 8.68 µl Hoechst 7.1 pH, incubated at 34°C for 30min
5. 200 x10^6 sperm/mL,10.54 µl Hoechst 7.1 pH, incubated at 34°C for 30min
6. 200 x10^6 sperm/mL,12.44 µl Hoechst 7.1 pH, incubated at 34°C for 30min
7. 100 x10^6 sperm/mL, 4.34µl Hoechst 7.1 pH, incubated at 34°C for 60min
8. 100 x10^6 sperm/mL, 5.27 µl Hoechst 7.1 pH, incubated at 34°C for 60min
9. 100 x10^6 sperm/mL, 6.22 µl Hoechst 7.1 pH, incubated at 34°C for 60min
10. 100 x10^6 sperm/mL, 4.34 µl Hoechst 7.1 pH, incubated at 34°C for 30min
11. 100 x10^6 sperm/mL, 5.27 µl Hoechst 7.1 pH, incubated at 34°C for 30min
12. 100 x10^6 sperm/mL, 6.22 µl Hoechst 7.1 pH, incubated at 34°C for 30min

Each stained sperm sample was diluted to 75 x10^6 sperm/mL with KMT, 0.75 µl/mL 5% red food dye. High dilution samples were then prepared by addition of 3mL KMT and 22mL 5mM HBGM-3 into 234µl stained sperm cell sample (at 75 x10^6 sperm/mL). Each stained sperm cell sample was then evaluated for % dead and resolution by flow cytometry analysis, and motility was evaluated in KMT and KMT,

2mM caffeine.

High dilution samples were then prepared by addition of 3mL KMT and 22mL 5mM HBGM-3 into 234µl stained sperm cell sample (at 75 x10^6 sperm/mL) and incubated at RT for about 1.5 hr. High dilution sperm cell samples were then evaluated for motility in KMT and in KMT, 4mM caffeine

Now referring primarily to Figure 12, it can be understood that sperm cells remain more viable when sperm cell concentration during staining is at about 100 M/mL versus 200M/mL without loss of resolution.

Certain embodiments of the sperm cell process system invention can further include the step of diluting sperm cells obtained from a male of a species of mammal to between about 75 M/mL and 200 M/mL to obtain a concentration of sperm cells that reduces, minimizes, or in which % dead in the sample after staining does not decrease with further increase in dilution of the sperm cells. Specifically, with respect to some embodiments of the invention the concentration of sperm cells can be less than 200M/mL and with respect to equine sperm cells can be about 100M/mL to reduce the number of % dead as evaluated by flow cytometry subsequent to the above described staining procedure.

Now referring primarily to Figure 13, it can be understood that as stain concentration increases fewer sperm cells survive and resolution decreases.

Now referring primarily to Figure 14, it can be understood that stain time can be substantially decreased without loss of resolution between X-chromosome bearing populations and Y-chromosome bearing populations of sperm cells evaluated by flow cytometery.

As such, embodiments of the invention can further include the step of decreasing

the stain concentration used in the stain protocol described above until the % dead in the stained sperm cell samples does not substantially decrease further, and can further include the step of decreasing the stain concentration used until the resolution of X-chromosome bearing sperm cell from Y-chromosome bearing flow cells yields a sorted sperm cell sample of less than 60% purity; or less than the % purity necessary or desired, such as 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%,94%, 95%, 96%, 97%, or 98%; or less than that which can be achieved for sperm cells from that species of male mammal; or not less than flow sorting one of or both of X-chromosome bearing or the Y-chromosome bearing from one another at a sort rate of not less than between about 500 sorts/sec to about 1000 sorts/second, between about 750 sorts/sec to about 1250 sorts/second, between about 1000 sorts/sec to about 1500 sorts/sec; between about 1250 sorts/sec to about 1750 sorts/sec; between about 1500 sorts/sec to about 2000 sorts/sec; between about 1750 sorts/sec to about 2250 sorts/sec; between about 2000 sorts/sec to about 2500 sorts/sec; between about 2250 sorts/sec to about 2750 sorts/sec; between about 2500 sorts/sec to about 3000 sorts/sec; between about 2750 sorts/sec to about 3250 sorts/sec; between about 3000 sorts/sec to about 3500 sorts/sec; between about 3250 sorts/sec to about 3750 sorts/sec; between about 3500 sorts/sec to about 4000 sorts/sec; between about 3750 sorts/sec to about 4250 sorts/sec; between about 4000 sorts/sec to about 4500 sorts/sec; between about 4250 sorts/sec to about 4750 sorts/sec; between about 4500 sorts/sec to about 5000 sorts/sec.

EXAMPLE 8. INSEMINATION OF COW ELK WITH SEXED FROZEN SEMEN

Cow elk 3-6-yr of age in Colorado and Minnesota were synchronized for estrus in September by insertion of a progesterone CIDR into the vagina for 12-14 d. Upon removal of the CIDR, 200 IU of eCG was administered intramuscular and elk were timed-inseminated 60 h later. Fresh semen was collected via electro-ejaculation from a 5-yr old bull elk and slowly cooled over 4 h to about 20°C for transportation as a neat ejaculate to the sperm-sorting laboratory. The ejaculate was concentrated to 1 x 10^9 sperm/ml for straining by centrifuging 1.5 ml aliquots for 10 sec at 15,000 x g. Semen

was incubated in 112 μM Hoechst 33342 at 200 x 10^6 sperm/ml in a TALP medium for 45 min at 34°C, and then diluted to 100 x 10^6/ml for sorting. Sperm were sorted on the basis of differing DNA content of X and Y chromosome-bearing sperm. X chromosome-bearing elk sperm contained 3.8% more DNA than Y chromosome-bearing sperm. Sperm were flow-sorted over a 4 h period using MoFlo®SX operating at 50 psi with a TRIS-based sheath fluid. The 351 and 364 bands of an argon laser, emitting 150 mW, excited Hoechst 33342 dye bound to DNA. Both X and Y chromosome-bearing sperm were collected (~92% purity as verified by reanalyzing sonicated sperm aliquots for DNA) were collected at ~4,700 sperm/sec into tubes containing 2 ml of 20% egg yolk-TRIS extender. Sorted volumes of 15 ml were sequentially collected. Approximately 110 x 10^6 sperm of each sex were sorted and cooled to 5 °C over 90 min. An equal volume of glycerol (12%) containing extender was added to the sorted volume at 5 °C. Sorted sperm aliquots containing 30-ml were concentrated by centrifugation at 4 °C for 20 min at 850 x g. Sperm pellets were pooled, adjusted to 21.7 x 10^6 sperm/ml and loaded into 0.25-ml straws. Each straw, containing 5 x 10^6 total sperm, was frozen in liquid nitrogen vapor. As a control, 5 x 10^6 total sperm from the same ejaculate were frozen in 0.25 ml straws at the same time as the sexed sperm. After thawing for 30 sec at 37 °C, 65% and 60% of sperm (control and sexed, respectively) were progressively motile as determined by visual estimates. Cows at 3 different locations and management schemes were inseminated using routine trans-cervical semen deposition in the uterine body. Pregnancy was determined 40-d post insemination by assaying blood for Pregnancy-Specific Protein B (Bio Tracking, Moscow, Idaho). Ten cows at one location were in poor condition at the time of insemination and no pregnancies were achieved with sexed or control sperm. The pregnancy rate at the other locations with sexed sperm (61%; 11/18) was similar to that for control inseminates (50%; 3/6). These pregnancy rates (sexed and controls) resulted from fewer sperm than are used in normal elk artificial insemination. Nine of eleven (82%) of sexed calves were of the predicted sex.

The invention can further include a mammal produced in accordance with any of the above described embodiments of the invention, or can include a mammal of predetermined sex in accordance with the various embodiments of the invention that

provide sperm cell insemination samples having an enriched population of either X-chromosome bearing sperm cells or enriched population of Y-chromosome bearing sperm cells, or a mammal produced in accordance with any embodiment of the invention in which a sperm cell insemination sample containing a low number of sperm cells compared to the typical number used to inseminate that particular species of mammal is used, or elk progeny produced in accordance with the invention as described above.

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves both a sperm cell process system including both techniques as well as devices to accomplish sperm cell processing. In this application, various sperm cell processing techniques are disclosed as part of the results shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways. Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this disclosure.

The discussion included in this provisional application is intended to serve as a basic description. The reader should be aware that the specific discussion may not explicitly describe all embodiments possible; many alternatives are implicit. It also may not fully explain the generic nature of the invention and may not explicitly show how each feature or element can actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in device-oriented terminology, each element of the device implicitly performs a function. Apparatus claims may not only be included for the device described, but also method or process claims may be included to address the functions the invention and each element performs. Neither the description nor the terminology is intended to limit the scope of the claims which will be included in a full patent application.

It should also be understood that a variety of changes may be made without departing from the essence of the invention. Such changes are also implicitly included in the description. They still fall within the scope of this invention. A broad disclosure encompassing both the explicit embodiment(s) shown, the great variety of implicit alternative embodiments, and the broad methods or processes and the like are encompassed by this disclosure and may be relied upon when drafting the claims for the full patent application. It should be understood that such language changes and broad claiming will be accomplished when the applicant later (filed by the required deadline) seeks a patent filing based on this provisional filing. The subsequently filed, full patent application will seek examination of as broad a base of claims as deemed within the applicant's right and will be designed to yield a patent covering numerous aspects of the invention both independently and as an overall system.

Further, each of the various elements of the invention and claims may also be achieved in a variety of manners. This disclosure should be understood to encompass each such variation, be it a variation of an embodiment of any apparatus embodiment, a method or process embodiment, or even merely a variation of any element of these. Particularly, it should be understood that as the disclosure relates to elements of the invention, the words for each element may be expressed by equivalent apparatus terms or method terms -- even if only the function or result is the same. Such equivalent, broader, or even more generic terms should be considered to be encompassed in the description of each element or action. Such terms can be substituted where desired to make explicit the implicitly broad coverage to which this invention is entitled. As but one example, it should be understood that all actions may be expressed as a means for taking that action or as an element which causes that action. Similarly, each physical element disclosed should be understood to encompass a disclosure of the action which that physical element facilitates. Regarding this last aspect, as but one example, the disclosure of a "flow-sorter" should be understood to encompass disclosure of the act of "flow-sorting" -- whether explicitly discussed or not -- and, conversely, were there effectively disclosure of the act of "switching", such a disclosure should be understood to encompass disclosure of a "flow-sorter" and even a "means for flow-sorting" Such changes and alternative

terms are to be understood to be explicitly included in the description.

Any acts of law, statutes, regulations, or rules mentioned in this application for patent; or patents, publications, or other references mentioned in this application for patent are hereby incorporated by reference. In addition, as to each term used it should be understood that unless its utilization in this application is inconsistent with such interpretation, common dictionary definitions should be understood as incorporated for each term and all definitions, alternative terms, and synonyms such as contained in the Random House Webster's Unabridged Dictionary, second edition are hereby incorporated by reference. Finally, all references listed in the list of References To Be Incorporated By Reference In Accordance With The Provisional Patent Application or other information statement filed with the application are hereby appended and hereby incorporated by reference, however, as to each of the above, to the extent that such information or statements incorporated by reference might be considered inconsistent with the patenting of this/these invention(s) such statements are expressly not to be considered as made by the applicant(s).

Thus, the applicant(s) should be understood to claim at least: i) each of the sperm cell processing devices as herein disclosed and described, ii) the related methods disclosed and described, iii) similar, equivalent, and even implicit variations of each of these devices and methods, iv) those alternative designs which accomplish each of the functions shown as are disclosed and described, v) those alternative designs and methods which accomplish each of the functions shown as are implicit to accomplish that which is disclosed and described, vi) each feature, component, and step shown as separate and independent inventions, vii) the applications enhanced by the various systems or components disclosed, viii) the resulting products produced by such systems or components, and ix) methods and apparatuses substantially as described hereinbefore and with reference to any of the accompanying examples, x) the various combinations and permutations of each of the elements disclosed, and xi) each potentially dependent claim or concept as a dependency on each and every one of the independent claims or concepts presented. In this regard it should be understood that for practical reasons and so as to

avoid adding potentially hundreds of claims, the applicant may eventually present claims with initial dependencies only. Support should be understood to exist to the degree required under new matter laws -- including but not limited to European Patent Convention Article 123(2) and United States Patent Law 35 USC 132 or other such laws-- to permit the addition of any of the various dependencies or other elements presented under one independent claim or concept as dependencies or elements under any other independent claim or concept. Further, if or when used, the use of the transitional phrase "comprising" is used to maintain the "open-end" claims herein, according to traditional claim interpretation. Thus, unless the context requires otherwise, it should be understood that the term "comprise" or variations such as "comprises" or "comprising", are intended to imply the inclusion of a stated element or step or group of elements or steps but not the exclusion of any other element or step or group of elements or steps. Such terms should be interpreted in their most expansive form so as to afford the applicant the broadest coverage legally permissible.